The Marabou meeting on the role of miRNA in nutrition and disease.

Table of Contents **Page No.**

Summaries of principal speakers' presentations

Philip James

This interactive discussion aims to provide the readers with an overview of the thinking at the time of the meeting in June 2013. First, we present a simple summary of the key speakers contributions presented in both an evening session and then in a full morning's series of presentations. There then followed a full afternoon's discussion with a recording of the points made with a further morning's discussion to conclude the meeting. Both the presenters and discussants contributions were obtained as a verbatim record and then I have produced the summaries and edited and rearranged to bring the different topics discussed into a more coherent format. The commentary also now has references included where these might be considered helpful. However, it is important to note that none of the contributors have reviewed this summary so their edited statements should not be cited as theirs but are provided as an aid for readers to gain some insight into this rapidly advancing field. Please also note that the presenters have also published a separate overview of the field with data added up to the end of 2014. [1]

Professor David Baltimore was finally unable to attend the meeting but gave his opening presentation and interacted by videoconferencing after his evening lecture with the other contributors. His initial presentation introduces the concepts and there follows a very brief summary of his presentation.

1. An overview of non - coding RNA with an extended focus on miRNA

David Baltimore:

MicroRNAs (miRs) are newly-appreciated key players in regulatory biology. This is particularly evident in the immune system where miRs act at many regulated nodes in the differentiation tree. We have studied in some detail miR146a, known from our earlier work to negatively regulate NF-k B, and our main tool is a mouse model in which we have knocked out the gene that encodes the miR. The consequences of that knockout are profound, ending after 8 months with bone marrow aplasia and after more than a year with myeloid cancer. We have traced the pathology to an overproduction of NF-kB -mediated cytokines. This occurs in the bone marrow where short-term hematopoietic stem cells produce excess cytokines because of a lack of resolution of the repeated inflammatory insults. This example shows how profoundly important miRs can be in maintaining homeostatic regulation. They are therefore sure to interact in all physiological systems and with other influences like nutrition.

The generation of miRNAs

The full extent of these RNAs and the many functions that they represent are only just beginning to become clear and I want to emphasise some of the characteristics that they share. MiRNAs are made by polymerase 2 from precursors and are regulated not only like protein coding genes, but they also have their own layers of regulation. Thus the control of miRNAs can be very precise. MiRNAs are made as direct copies from DNA in a precursor form i.e. pri-miRNAs. In these precursors there are little hairpin regions and the drosha protein picks out these hairpin regions, cuts them clean from the precursor RNA and allows them to be exported to the cytoplasm where Dicer cuts off the loop on the end of the double strand generating a small duplex of about 22 nucleotides. Then one strand of that is loaded into a protein complex called the RISK complex in a very precise way so that the hydrogen

[1] Nolte-'t Hoen EN, Stoffel M, Van Rooij E, Bushell M, Zhang C-Y, Dashwood R, James WPT, Harris C, and Baltimore D. The role of MicroRNA in nutritional control. Journal Internal Medicine.2015; doi:10.1111/joim12372

bonding potential of the bases is open. Then in the RISK complex, the binding of the 5 prime end of the miRNA interacts with the so-called seed sequence of 6-8 nucleotides in the 3'UTR of the message. This is sufficient to provide tremendous specificity although any given miRNA can react with a whole variety of messenger RNAs. These messenger RNAs are then prevented from translating proteins and are themselves degraded.

The control of haematopoiesis and inflammation.

We study miRNAs in the context of the haematopoietic series which, of course, includes all the blood elements, i.e. the blood cells, that are derived from a haematopoietic stem cell. The haematopoietic stem cell can give rise either to progeny that become lymphoid cells or myeloid cells. And among the myeloid cells are the macrophages and granulocytes, as well as erythrocytes and megakaryocytes that are then on a somewhat separate lineage. Now in these evolving pathways for generating different cellular entities there is a whole series of miRNAs each one of which is known to play a role both in determining the route and the rate and form of development at separate points in this haematopoietic tree. They also affect the function of these cells. The density of the known miRNAs is increasingly evident. We are familiar with the idea of transcription factors, historically considered to be the controllers of gene expression, but miRNAs interact in the same ways, controlling these complicated routes of differentiation and function.

We have studied miRNAs as important parts of the regulatory circuitry of immune cells. NF-kB is a transcription factor which is a key player in inflammation and immune responses. NF-kB resides in a quiescent state in the cytoplasm until there is an inflammatory stimulus e.g. tumour necrosis factor, lipopolysaccharide etc. which releases NF-kB from its inhibitor and allows it to transfer to the nucleus there to induce the transcription of many genes coding for hundreds of proteins. Among these induced genes are genes that encode miRNAs one of which is miRNA-146a.

We looked some years ago now at which miRNAs might be stimulated by NF-kB and found three of them, 132, 146 and 155. The precursors for miRNA-146A and 155 are processed to their miRNAs. MiRNA 155 activates immune responses in a complex way whereas miR-146 interacts with the mRNAs for two proteins, I-RAC1 and TRAF-6 - mainly we think now TRAF-6. These proteins are, however, interacting with the signal transduction pathway that activates NF-kB, and down-regulates NF-kB production. Thus TRAF-6 is a feedback negative regulator of the NF-kB pathway. This is an important metabolic control for ensuring that the stimulus to the production of many proteins by NF-kB is not long lasting because otherwise molecules such as the cytokines can have drastic effects. Some years ago we knocked out the gene for miRNA 146a and found that the mice were born normal and seemed to develop normally but over the following 6 months they developed myeloid infiltration of the spleen and other organs; over the subsequent year they developed a myeloid cancer. So miR-146a plays a critical role in maintaining the appropriate pattern of myeloid development as well a role in suppressing myeloid cancer. All of these pathologies do not emerge if we also knockout one of the sub-units of NF-kB. So it is NF-kB control which is the apparent cause of this pathology.

Jimmy Zhao in our lab studied the role of miRNA 146a as a guardian of the quality and longevity of haematopoietic stem cells. He purified a mixture of all the undifferentiated precursors i.e. the long-term stem cells, the short-term stem cells and the multipotent progenitor (MPPs) stem cells by a negative selection process operating against their content of lineage markers thereby ridding the mixture of all the mature cells. This provided him with about 1% of bone marrow cells. Then he pulled out the true long-term haematopoietic stem cells from that mixture by using a positive selection for the CD150 marker and a negative selection for CD48. If we then take the miRNA 146a knockout animals at 6 weeks of age and purify their haematopoietic stem cells and their precursors, we found them to be normal but by 4 months of age we found an increased numbers of myeloid cells, an increase in the haematopoietic precursor cells and even an increase in stem cells. So the bone marrow

seemed to be working overtime. Yet by 8 months old, we see a severe depletion of all of these cell types; the haematopoietic stem cells are clearly losing functionality, even before we see the loss of their physical presence.

Of all the proteins induced by NF-kB activation IL-6 stands out as playing a key role. So we interpret this being a pathological state of chronic inflammation occurring over several months. This, if true, should mean that lipopolysaccharide would accelerate these processes. By using a fluorescent reporter gene that detects the presence of activated NF-kB we were able to show that normal 2 month old animals have only 7% of their Lin(-)/c-Kit (+)/Sca-1 (LKS) cells and 7% of the haematopoietic stem cells showing activation whereas if treated with lipopolysaccharide for a few days 30% of both cell types are activated. Then if we go to older animals that have not been purposely activated, we see about 17% of the cells are activated, implying that ageing as well as inflammatory stimuli can activate the NF-kB in these cells. So we have concluded from a number of experiments that IL-6 is the main mediator of this activation and that the single miRNA 146a is an absolutely critical regulator of haematopoietic stem cell longevity and quality through the ageing process of animals and through the many insults of chronic inflammation that animals suffer. MiRNA 146a is not the only regulator, but it is a key regulator. It does this by blocking the TNF receptor associated factor (TRAF)6 which then blocks the induction of NF-kB and thereby prevents activated NF-kB stimulating IL-6 production and this in turn saves the haematopoietic stem cell from over-stimulation.

This pathway can be summarised when miR-146 blocks the TRAF induction of the inflammatory sequence as:

miR-146a →TRAF-6 →NF-kB→IL-6 →HSC (haematopoietic stem cells)

Then we used specially developed techniques in conjunction with James Heath at Caltech to study the origin of the cytokines. Heath's student, Chao Ma, working closely with Jimmy Zhao in our lab, was able to identify single cytokines by micro techniques and show that there are single cells which secret their own specific cytokine. The first result observed was that the haematopoietic stem cells, no matter whether they are stimulated or not, secrete nothing. So although haematopoietic stem cells (HSC) do have NF-kB, and could in principle respond they do not. The Lin(-)/c-Kit (+)/Sca-1 (LKS) cells, one form of the haematopoietic stem cells (HSCs), are quite responsive with 6% of them secreting lipopolysaccharide (LPS) alone, and 20% secreting lipopolysaccharide + Plasma membrane associated membranes (PAM); 40% of these dual-stimulated cells secrete at least one cytokine, although the HSCs don't produce any. So we have uncovered here a differentiation step from the true haematopoietic stem cell to a cell which can give rise to all of the various blood cell progeny, but can also be activated to secrete cytokines in response to inflammatory stimuli like LPS and PAM. If you look at the LKS cells, they secrete large amounts of all sorts of cytokine whereas myeloid cells are the only other ones that make significant amounts of many different cytokines. The lymphoid cells don't make much of anything. So what we are seeing quantitatively as well as in terms of the breadth of secretion potential is that these LKS cells are super-secretors.

Now when the miRNAs are knocked out, a much greater reaction to inflammatory stimuli develops, but a population of double knock out cells, where NF-kB is also knocked out, shows only 5% of cells secreting cytokines which we have shown to be bioactive. If you just knock out NF-kB, almost nothing secretes even though the cells have been stimulated.

So infection is a process which is not only sensed peripherally, at the sites of infection, but probably by the carriage of activators such as lipopolysaccharide in the circulation which then affect the marrow. There the miRNA146a should be seen as a guardian against pathogenic stimulation and the limiting controller of the response to what are called PAMPS - pathogen associated molecular patterns.

Human blood disorders.

Myelodysplasia, a really serious disease in humans which is moderately widespread, looks very similar to what we have been seeing in these 146a knockout animals. The myelodysplastic syndrome (MDS) involves a loss of bone marrow function, a wiping out of bone marrow cells and ultimately converts into myeloid leukaemia in most patients who suffer from it - this is replicated in our mice studies.

So let me generalise. There are something like 500 miRNAs in the human genome: the number remains under debate - and if each has a role anywhere near as important as the miRNA146a, then one can assume that these miRNAs have very important roles. Eric Olsen's work has shown the important role of miRNAs in the heart; in the liver many investigators have also found important miRNAs and although we have to recognise that many miRNAs may be highly cell-specific in their action miRNAs - even 146a - turn up in lots of other kinds of cells and in each cell and we really need to understand what are its targets and what is their physiological roles?

Discussion:

Mikael Rydén: We have been looking at miRNA regulation before and after weight loss for instance, and trying to identify miRNAs important for cytokine production. What do you think of the role of miRNA 146a in humans? You alluded to its possible importance in myelodysplastic syndrome (MDS) of the bone marrow: have you, or anybody else, looked at the 146a expression in MDS subjects? And on the therapeutic side, what about increasing the expression of 146a? Can you rescue the effect in the knockouts by giving exogenous 146a for instance?

David Baltimore. Yes. We find a reduced 146a in the cells of MDS patients: obviously in some patients more than in others. We compare it to normal marrow and we have also compared it with acute myeloid leukaemia (AML) cell populations and again it is lower than you see in AML. So it does satisfy that criterion, but I don't like the experiment because when you compare MDS cells, normal marrow and AML cells, you are comparing apples, oranges and pears. There are different cell populations so we may be looking at differences that relate more to the nature of the cells than to the pathologic process. So I think it takes a lot more study to find out what is going on, but at least it is consistent.

We have not attempted to overcome the knockout with exogenously provided 146a - it is difficult to do an experiment like that - I work with one of several companies that are trying to use antagomirs, antagonists of miRNAs, as well as miRNA mimics to try to affect physiology. That involves a long term effort with very interesting but complex chemistry to generate molecules that you can actually use therapeutically - I think we will see a lot of that over the next decade.

What we have done is over-express 146a in marrow in a normal wild type of mouse and we found immunosuppression but incidentally if we do this with miRNA155, we get immune activation which shows you that these two are really working in opposite directions.

MiRNA146b's role?

Eva van Rooij: Can you tell us something about the expression and function of miRNA 146b and where it occurs?

Baltimore. The 146 b is expressed in some of the same cell types, although I think at lower levels, and is not as responsive to induction. We are currently studying its role.

Ageing?

Howard Chang. It seems that your haematopoietic stem cells are long lived and this raises the issue of ageing. It seems to take time for NF-kB to get activated. Is the effect uniform or

can one detect variation in activation with time implying that there is ageing in the responsiveness of stem cells?

Baltimore. Your own work on the ageing of stem cells is extremely interesting[1]. Our problem is that we cannot recover as yet the cells we are testing in the assay wells so we can't know that a cell is secreting or not secreting and then recover it and test its ability to repopulate animals or to respond further to cytokines or anything else at the moment. So you are right - we have only looked at activation at the moment.

Nutrition and biological variation?

Robert Chapkin. When you show increases in the IL-6 during activation not only is there an increase in the mean levels but a much greater variation. This a feature of many metabolic systems but why in this case is the variation also affected? Then there is the question as to why poorly nourished and infection prone children grow slowly. Are their stem cells dividing more slowly or are some stem cell populations lost?

Baltimore. Again we are unable to recover the cells to test the basis for this variation and I just do not know what the stem cell response is in malnourished children***.***

Martijn Nolte. You showed that the miRNA 146a knock- out mice had a very large response in IL-6 - much greater than the wild type could produce so is IL-6 having an autocrine effect?

Baltimore. Our experiments are rudimentary at present. We have done studies adding pure IL-6 and do not get an effect so there may be more than one cytokine involved.

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2. An evolutionary approach to miRNA

Ester Nolte-'t Hoen

It is known that the number and the repertoire of protein coding genes remains relatively stable across the genetic spectrum of the multi-cellular species despite the fact that their complexity and development and cognition increases markedly. Not too long ago we thought that our DNA contained a lot of junk DNA but we now recognise that a large proportion of the DNA allows the generation of molecules which are non-coding RNAs that have the regulatory power to allow human evolution, development and even cognition. These molecules, which do not code for proteins, can be crudely divided into housekeeping RNAs and the regulatory large and small non-coding RNAs. These housekeeping RNAs e.g. the ribosomal RNAs (rRNA) provide the ribosomal structure, the transfer RNAs (tRNA) that enable the translation of messenger RNAs to produce proteins, small nuclear (snRNA) that are involved in splicing and small nucleolar RNAs (snoRNAs) which guide the modification of other RNAs by methylation and pseudouridylation (psudouridine is an isomer of uridine) and which thereby modify RNA structures and are known to be involved in the basic function and viability of cells Then there are the small non - coding RNAs micro RNA (miRNA), small interfering RNAs (siRNA) and piwi interacting RNA (piRNA) that play a regulatory role and are usually expressed at certain stages of development or cell differentiation and may also be induced by external stimuli. There are also many other small non coding molecules containing RNA which are now known to have a wide range of highly complex regulatory functions with counterbalancing effects.

The role of miRNAs

The miRNAs operate by pairing their bases with complementary sequences within messenger RNA (mRNA) molecules and thereby either induce a cleavage of the mRNA, reduce its efficiency of translation or destabilise the mRNA . These multiple types of miRNA target the majority of human genes so can have a powerful regulatory effect on gene expression. Because the specific sequences of the miRNA can target multiple mRNAs and as several miRNAs are also capable of targeting the same messenger RNA this suggests that these miRNAs may act in a counterbalancing way to ensure that a regulatory step is not

over-activated or suppressed. This means that their role may only become obvious under stress conditions. Several key signalling pathways are also now known that regulate not only the transcription of miRNA but also its biogenesis pathway so this is another regulatory process.

MiRNA cell secretion

Cells can regulate the levels of small encoding RNAs in other cells by releasing extracellular vesicles containing RNA. This is then a means of communication in addition to the well-known signalling mechanisms involving receptors for hormones and the secretion of chemokines and cytokines. These extracellular vesicles may arise from within the endoplasmic membrane and fuse with the plasma membrane with the subsequent release of small vesicles i.e. exosomes which are packages of both messenger RNAs and miRNAs. They are generally around 100 nanometres in size and consist of a bi-layer containing membrane proteins within which there are lipids, luminal proteins and the genetic material which can then selectively target other cells for the transfer of its constituent molecules. In these target cells novel genes can then be expressed and regulate gene expression. The proteins and lipids within the exosome help to selectively target recipient cells and help in the uptake of the exosomes. There seems to be a huge variety of extracellular vesicles containing miRNA but these vesicles also contain an even greater variety of other small encoding RNAs but with specific rather than all types of miRNAs. Transfer RNAs (tRNAs) are particularly dominant in vesicles rather than in cells and some components or fragments of the tRNA are now found as separate entities with increasing evidence of their specific cellular functions. Amongst the other forms of RNA in these exosomes are vault RNAs which are cytoplasmic organelles which are called vault RNAs because of their appearance under an electron microscope - they look like a vault with a 3-fold symmetry within a cell. They are present in many types of eukaryotic cells and are highly conserved and form part of the lipid rafts in cells.

It has also become clear that the number and type of extracellular vesicles present in body fluids can reflect the health or disease status of the tissues and that makes these vesicles very good biomarkers for the detection and monitoring of disease. Furthermore some types of extracellular vesicles may turn out to be useful in therapeutic terms by allowing the selective targeting of both tissues and particular molecular mechanisms.

Exosomal miRNA: nutritional implications

We and others have found many extracellular vesicles in human breast milk which is a very complex fluid known to transfer passive immunity to the neonates by transferring ready-to-use immune components such as immunoglobulins and antimicrobial molecules. But it is also known that some of the components transfer active immunity and instruct the immune system of the child. For example, they seem to instruct which antigens to generate immunity to and to which antigens to induce tolerance. When we look at the membrane enclosed components of breast milk we find many different cell types as well as the milk fat globules which are the main energy storage units in milk. The milk includes in great abundance extracellular vesicles derived probably from a variety of the mother’s cells so a neonate ingests hundreds of millions of cells per day.

Some of these cells can traverse the intestinal epithelial layer and have been detected in the child's systemic circulation up to 9 days after delivery. Their specific functions are still unknown but our hypothesis is that milk extracellular vesicles can have an even more important and widespread role in signalling to the child because these vesicles are much more robust than the ordinary cells found in the breast milk. The exosomes are so small that they can easily traverse the epithelium layer and thereby transfer specific proteins and RNAs into the offspring. So these exosomes might be involved in instructive signalling to the immune system of the child.

Preliminary discussion

Mikael Rydén: I was wondering about these extracellular vesicles and their stability in milk. What happens when you pasteurise cow's milk for instance?

Ester Nolte-'t Hoen: We don't know yet.

Chen-Yu Zhang: Are these exosomes derived from the membrane of cells or are they derived from the endosomal system?

Ester Nolte-'t Hoen: That is a major question at present. You could say that the larger vesicles are probably derived from the plasma membrane but the smaller vesicles could come from either source.

Martin Bushell: Is there any evidence to support that there is particular targeting with miRNA from one tissue to another tissue at a long-distance within the body?

Ester Nolte-'t Hoen: I'm not sure - we have been studying adjacent targeting so far.

Cutberto Garza: Human milk's immunological profile is very dynamic. It changes incredibly from colostrum to transitional milk to mature milk. Which of the milks have you been looking at in terms of its vesicular profile?

Ester Nolte-'t Hoen: Although the composition of milk is changing all the time it is not known if extracellular vesicles are also changing but one would expect so.

Olle Hernell: There are also bacteria in milk and some of these may be probiotic bacteria rather than contaminants and actually affecting colonisation of the gut in the infant. How much of the vesicles come from bacteria?

Ester Nolte-'t Hoen: We, of course, exclude bacteria as such in our analyses but bacteria also produce miRNA exosomes as you say so this still needs to be studied.

3. Overview of miRNA control of metabolism

Markus Stoffel: We recognise that we change our metabolism cyclically during the day with meals being eaten followed by fasting at night as we change from an anabolic to a catabolic state. These metabolic cycles are mainly mediated by hormonal cues signalling through specific signalling pathways in the target organs and impinge on transcription factors which change gene expression. There is a layer of regulation on top of this regulatory control system mediated in part by miRNAs. There are a number of ways whereby these regulatory networks are stabilised by miRNAs through signal modulation, negative and positive feedback routes, but also by an overall buffering of gene expression. This buffering function means that if one considers model organisms where single miRNA animal are knocked out then this does not lead to a specific new phenotype. However, if these organisms are stressed in a specific way, this is when we see in miRNA knockouts specific phenotypes emerging because the regulated networks normally buffered by the miRNA are perturbed so much that we can then see new phenotypes.

So I want to start with miR-122 because this is certainly a very special miRNA which in the liver is highly expressed with approximately 70,000 copies per cell. With colleagues in industry we devised an antisense RNA to inhibit the miRNAs in the liver. Now the liver is involved in a multitude of pathways including cholesterol synthesis and we found that miR-122 regulates the gene expression of cholesterol synthesis without changing glucose or lipid metabolism or many other functions; targeting the miR-122 led to a 44% reduction in cholesterol synthesis[2]. This is an example of a highly expressed miRNA involved in cholesterol metabolism which has now been shown to be active in 7 different species, including non-human primates[3] and humans[4]. However, we also found a range of miRNAs involved in general metabolism.

One illustration of the potential wide ranging actions of a single miRNA is that miR-221 not only affects cholesterol metabolism but is also critical for the replication of some viruses e.g. hepatitis C. So by silencing miR-221 there is a very marked inhibition of virus replication. This observation is now being tested therapeutically in Phase 2 trials.

An example of the range of crucial metabolic actions controlled by miRNA in the liver relates to miR-802 which is found to be up-regulated in livers of different models of human disease and nutritionally induced abnormalities. So the Brüning group published recently an analysis showing that a high fat diet induces an increase in miR-802 in the liver and it is even more markedly increased in diabetic db/db mice[5]. Transgenic overexpression of miR-802 leads to marked glucose intolerance and if one silences the miRNA then one induces increased insulin sensitivity in the mice. The expression of miR-802 is overexpressed in obese humans so this is also a conserved metabolic process[4]. The mechanism involves the miR-802 targeting a specific sequence, tcf2 which used be called hepatocyte nuclear factor 1β. This gene is also known as a maturity onset diabetes of the young (MODY) 5 gene which is a relatively uncommon autosomal dominant inherited gene of the MODY group of inherited conditions ; these children are not just diabetic but have other kidney and pancreatic cell abnormalities. Interestingly, a number of investigators have shown that young people with mutations in tcf1 have increased hepatic insulin resistance which is relatively unusual for MODYs who typically have pancreatic cell defects.

Another example of hepatic metabolic control by miRNAs came from studies we undertook on miR-103 and miR-107[6]. We showed that the expression of microRNAs 103 and 107 is upregulated in obese mice. Silencing of miR-103/107 leads to improved glucose homeostasis and insulin sensitivity. In contrast, gain in miR-103/107 function in either liver or fat is sufficient to induce impaired glucose homeostasis. We identified caveolin-1, a critical regulator of the insulin receptor, which sits in the plasma membrane of adipocytes and other cells. It stabilises the insulin receptor as a direct target gene of miR-103/107. We demonstrate that caveolin-1 is upregulated upon miR-103/107 inactivation in adipocytes and that this is concomitant with stabilization of the insulin receptor, enhanced insulin signalling, decreased adipocyte size and enhanced insulin-stimulated glucose uptake. These findings therefore showed the central importance of miR-103/107 in insulin sensitivity and also revealed a new potential target for the treatment of type 2 diabetes and obesity. We have also found these miRNAs are upregulated in obese humans. Experimentally we can silence these genes and improve glucose tolerance in all the models that we have looked at.

Fat metabolism, the control of brown adipocytes and energy production

Classically we think of white and brown fat cells, the former being known for storing energy as fat and the latter as an energy consuming, heat producing cell. Brown adipocytes (BAT) are also very effective in removing small dense LDLs from circulating lipoproteins in the blood. When an animal is exposed to the cold BAT cells are stimulated to produce heat and some white cells are transformed into multilocular adipocytes expressing UCP1, a mitochondrial protein that plays a key role in heat production by uncoupling the activity of the respiratory chain from ATP synthesis. These adipocytes have been named “brite” adipocytes. Spiegelman originally showed[7] that brown, but not white fat cells arise from precursors that express *Myf5*, a gene previously thought to be expressed only in the myogenic lineage. The transcriptional regulator PRDM16 controls a bidirectional cell fate switch between the development of either skeletal myoblasts or brown fat cells. Loss of PRDM16 from brown fat precursors causes a loss of brown fat characteristics and promotes muscle differentiation. Conversely, ectopic expression of PRDM16 in myoblasts induces their differentiation into brown fat cells. PRDM16 stimulates brown adipogenesis by binding to PPAR-γ (peroxisome-proliferator-activated receptor-γ) and activating its transcriptional function. Finally, PRDM 16-deficient brown fat displays an abnormal morphology, reduced thermogenic gene expression and elevated expression of muscle-specific genes. Taken together, these data indicate that PRDM16 specifies the brown fat lineage from a progenitor

that expresses myoblast markers and is not involved in white adipogenesis. The conversion of brown adipocytes to white adipocytes is also highly regulated. We found that miR-133 normally suppresses BAT metabolism but is itself suppressed when animals are exposed to cold and BAT is activated. MiR-133 directly targets and negatively regulates PRDM16, and inhibition of miR-133 promotes the differentiation of precursors from BAT and subcutaneous adipocytes to mature brown adipocytes, thereby leading to increased mitochondrial activity and energy production. Forced expression of miR-133 in brown adipogenic conditions prevents the differentiation to brown adipocytes in both BAT and subcutaneous adipose tissue precursors. So miR-133 and another factor Mef2 are central upstream regulators of Prdm16 and therefore control brown adipogenesis in response to cold exposure. What this all means is that if we can suppress miR-133 we may therapeutically increase energy expenditure by stimulating brown adipocyte activity.

Pancreatic function and diabetes

A decade ago we found that pancreatic beta cells contain many miRNAs but especially miR-375 which constitutes 10% of all the miRNAs in this cell type[8]. We showed that overexpression of miR-375 suppressed glucose-induced insulin secretion, and conversely, inhibition of endogenous miR-375 function enhanced insulin secretion. The mechanism by which secretion is modified by miR-375 is independent of changes in glucose metabolism or intracellular Ca2+-signalling but correlated with a direct effect on insulin exocytosis. Myotrophin was identified as a target for inhibition by miR-375 and controlled glucose-stimulated insulin secretion and insulin extrusion from the islet cells. Given the regulatory role of miR-375 in insulin secretion it obviously becomes a pharmacological target for the treatment of diabetes.

When miR-387 is suppressed insulin secretion is reduced and mild hyperglycaemia follows without any abnormal morphology of the islets. We have found that these mice are diabetic for 2 reasons: one reason is that these genetically manipulated mice with miR-375 suppressed also have a constitutive elevated glucagon which leads to chronic stimulation of the liver by glucagon and increasing glucose production[9]. The other reason for the mice's hyperglycaemia is that they have a smaller pancreatic β-cell mass and there are fewer β-cells. This was an interesting phenotype because in human type 2 diabetes there is a gradual decline in pancreatic β-cell mass. So we wanted to find out if this miRNA plays a role in the normal compensatory response of pancreatic β-cells. In humans pancreatic β-cell hyper-secretion of insulin will compensate for insulin resistance for the longest time if pancreatic beta cell mass increases and if metabolically there is an increased demand for energy e.g. by exercise. So we decided to cross the knockout into the ob/ob mouse which characteristically has huge islets as it compensates for the high inflow of food in the absence of leptin. The ob/ob mouse is only very slightly hyperglycaemic but when we knock out the miR-375 in the ob/ob mouse then the blood glucose rockets up and these mice die prematurely[9]. They die because they cannot compensate for the insulin resistance; their islets remain miniscule, sometimes consisting of a few cells and therefore are not able to produce enough insulin.

MiR-7 and the Central Nervous System

The miR-7 family of miRNAs are not only found in the pancreas but also in the central nervous system. These miRNAs are highly expressed in β-cells where they are down-regulated on a high fat diet and in obesity. However, when miR-7 is overexpressed then pancreatic cell hypoplasia develops and the mice become highly diabetic and their pancreases have a decreased insulin content because there is less insulin production: all the transcription factors that determine differentiation of the islets and direct the transcription factors for insulin production and secretion are all markedly down-regulated. But these pancreatic β-cells don't die, they are just quiescent. Interestingly recent analyses also show with imaging that patients with type 2 diabetes had a reduced pancreatic volume[10] so there

is now an opportunity with PET scanning to study the reactivation of islet cells in patients with diabetes.

Preliminary Discussion

Pier Paolo Pandolfi: I guess your knockouts were tissue specific but is it possible that some of the effects you see arise from exosome vesicular transfer of metabolic control from one organ to another?

Markus Stoffel: A good question. We need genetic models to answer this question of possible remote signalling from several organ systems. Other than the issue of milk miRNA we have heard about we had no evidence of miRNAs transfer from one tissue to another. In our knockouts of the miR-375 we have rescued the knockout with a transgenic specific expression only in the pancreatic β cells. We find no serum miRNA and detailed analyses of other organs also fail to show any specific miRNAs that are likely to be metabolic messages. MiR-375 is actually seen in breast milk and has a relatively high expression in the epithelium of the mammary gland. It is secreted in high amounts especially in the colostrum and we are currently designing a genetic experiment to show if the miR-375 transfers metabolically to the offspring.

Curt Harris: Your phenotypes that you are getting with your miR knockouts are quite interesting. Have you looked at the haplo-insufficiency of miR-375 because it acts as a tumour suppressor in some human epithelial cancers.

Markus Stoffel: Yes, we always find it a little bit higher than the wild-types but it is subtle.

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4. A cardiac miRNA with apparent effects on energy homeostasis.

Eva van Rooij

We have been studying a cardiac specific miRNA which also influences metabolic disease. The heart, of course, has to react to different demands e.g. exercise and it does this by remodelling and becoming larger. Cardiac cells are fully differentiated so there is no cell division in response to increased demand but hypertrophy of the same number of cells. This hypertrophy involves the re-expression of an array of foetal genes with the preservation of a balance between the two main contracting genes, the α myosin heavy chain and β myosin heavy chain. The α myosin heavy chain is the predominant myosin heavy chain gene in rodent hearts, but the β myosin heavy chain is the predominant myosin heavy chain in larger mammals, monkeys and humans. A shift towards β myosin heavy chain is considered a maladaptive change. Thyroid hormone is really a very important regulator of the balance of alpha / beta myosin heavy chain levels in the heart so whenever the alpha chain goes down, the beta chain goes up. In response to stress or hypothyroidism there is a shift towards β myosin heavy chain which we consider a pathological sign with a lower contraction capacity of the heart.

There are roughly 200 to 300 miRNAs detectable in the heart and we think that only a portion of these - around 50 - are actually relevant for cardiovascular biology. So far we have found miR-208 only in cardiac muscle but deleting it seemed to have no effect - a feature we might expect from the previous presentations emphasising the subtle effects of miRNAs. However, when the heart is stressed by using an obstructive aortic band to induce cardiac hypertrophy and the miR-208 is deleted then there is no β myosin induction and the heart is unable to hypertrophy.

MiR-208 controls a network of transcriptional repressors[11] as shown when we antagonize the action of miR-208 by producing a sequence which acts as an antagonist i.e. an antagomir. We also use the RNA analogue Locked Nucleic Acid (LNA) modified antimiR

which readily dissolves in physiological salts and this allows us to systemically deliver them subcutaneously[12]. Then when we tested this antagomir in salt sensitive hypertensive mice we inhibited the miRNA-208 and blocked the cardiac hypertrophy and fibrosis in response to the high-salt diet and improved survival. The endocardiac myosin shifted so the induction of β was blocked and the α-myosin heavy chain was brought back to normal levels[13]

When we then assessed the long-term effects of the antagomir to miR-208 we observed that the animals were not putting on weight as in the control group and one could block the high fat diet induced weight gain[14] and improve glucose tolerance. We had already shown that the miR-208 worked by targeting the α myosin heavy chain promoter MED 13 gene which encodes a component of the mediator complex known as TRAP, a transcriptional coactivator complex thought to be required for the expression of almost all genes. So we then over-expressed MED13 in cardio-myocytes thereby partially mimicking the miR-208 knockout animals. The transgenic animal's hearts appeared to be normal so there was again no detectable obvious pathological signs from over-expressing MED13, again in parallel with our findings with miR-208 knock-outs. But we then found these transgenic animals were far leaner than their wild-type counterparts. In response to a high fat diet the MED13 overexpressed animals did not get fatter and had a notable reduction in abdominal fat and much better glucose tolerance thereby implying a metabolic role for the cardiac specific miR-208.

We then applied the same techniques to rat models and to the Zucker rat which has the same defect in leptin receptor as the obese hyperphagic db/db mouse with similar cardiac hypertrophy. Again the antagomir to miR-208 limits the weight gain and improves glucose handling. We are now looking at a monkey model of fructose induced diabetes to assess the systemic role of the cardiac specific miR-208 but how this antagomir to miR 208 functions in energy homeostasis is still a mystery. Exosomes may be involved but the liver and kidney as well as other tissues will have been exposed to the antagomir during the experimental process.so whether there are other interactions remains to be established.

Preliminary Discussion

Leif Groop: Did you see any change in the muscle fibre composition in those animals where you saw a marked decrease in fat mass? Did they move more or increase energy expenditure in other ways?

Eva van Rooij: We saw no change in the heart fibre composition, but we did see it in the skeletal muscle. There is also a 208 isoform in skeletal muscle and if you inhibit that miRNA you will see a shift in myofibril content. The animals were certainly more active so there is also more oxidation in these hearts.

Kenneth Chien: Presumably there is some kind of hormone being secreted from the heart or elsewhere which then affects the tissues but is there any effect on thyroid hormone levels and is the miR- 208 only responsible for the stress change in the gene or is it the universal controller?

Eva van Rooij: We measured T3, T4 and we didn't see any change so it is not that simple. I think there is a thyroid influence for sure. We think that other tissues like skeletal muscle might be involved in this but whether by deleting MED13 we affect non-cardiac tissues we do not yet know. In developmental regulatory terms the α and β myosin balance does not come under thyroid regulatory control until a couple of days after birth.

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5. Role of miRNA in foetal programming

Martin Bushell. I will assume that you are all familiar with the developmental origins of disease hypothesis whereby early environmental factors e.g. diet programmes the metabolism of the offspring so that the offspring's lifelong responsiveness to environmental conditions is altered. I have been undertaking this work with Sue Ozanne from Cambridge UK.

We have used a rat model with the mothers being put on a low 8% protein diet as opposed to a 20% protein diet. The offspring from these mothers then are of low birth weight and grow with a smaller fat mass and smaller adipocytes when they are weaned normally onto a normal diet. Yet they develop type 2 diabetes at about 15 months with increased circulating levels of triglycerides and fatty livers. We used miRNA micro arrays on the offspring 21 days after birth and found a host of up-regulated and down-regulated miRNAs. We then looked for persistent, programmed changes in gene expression at 3 months after birth and we found a different array with one MiRNA, miRNA-483, showing marked up-regulation both at 21 days and at 3 months after birth. miRNA-9 was also down-regulated but we haven't investigated this so far.

The miRNA-483 gene is found within one of the introns of the insulin growth factor 2 (IGF2) gene. This IGF2 gene is an imprinted gene from the paternal allele and we, with others, have shown that miR-483 is also imprinted. The IGF2 is mainly expressed during foetal life when embryogenesis occurs and low birth weight children born under famine conditions have IGF2 imprinted differently. In our offspring of low protein fed pregnant rodents we can also confirm that the miR-483 gene is up-regulated (as assessed in adipose tissue) at about 21 days and at 3 months. To assess the relevance of this in humans we have compared the babies in the lowest decile of birth weights with those born with weights between the 50^{th} and 90^{th} centiles. The miR-483 was also up-regulating in the adipose tissue samples from low birth weight babies suggesting that these features are conserved across the species.

The target of this miRNA was unknown but we noticed that the growth and differentiation factor 3 - GDF3 from the TGFβ family is highly expressed in adipose tissue and when this GDF3 is overexpressed it increases the body fat mass. But when GDF3 knock - out mice are tested they have smaller adipocytes and can no longer become obese on a high fat diet. The conserved 3'UTR of GDF3 is very close to the stop codon and inhibits translation of this reporter construct. MiR-483 targets GDF3 and we found that GDF3 is down-regulated in the adipose tissue by about 40% in these offspring from low protein diet mothers. Interestingly, when we look at the human adipose tissue samples we also find that GDF3 is down-regulated substantially in those born small. So this looks like a really important conserved mechanism for potentially controlling adipose tissue differentiation. If we then take a model for adipose cell differentiation we find that the insertion of a mimic of miR-483 induces smaller adipocytes. In vitro studies strongly suggested that adipose cell differentiation was indeed limited and controlled by miR-483. So knocking out miR-483 induces larger adipocytes but we are not clear that it alters the number of adipocytes.

So what we are proposing here is that when the mother is put on a low protein diet then the offspring are being programmed to lay down less fat and perhaps limit adipose expandability. These changes are induced by increasing adipose tissue miR- 483 which decreases GDF3 and stops the ability of these adipocytes to expand and take up triglycerides. So triglyceride storage if needed in these programmed animals/individuals will have to be stored elsewhere e.g. in the liver, muscle etc. So this could then potentially link to insulin resistance, type 2 diabetes and metabolic disease. Finally in normal weight offspring of obese pregnant rodents we find a decrease in miR-483 levels which can then be expected to allow a far greater expansion in the adipocytes of the offspring of these obese mothers. This then is a mechanism for environmental factors such as excess energy intake feeding through and programming the offspring

Preliminary Discussion

Frank Slack: What maintains the sustained expression of this miRNA over months: is this an epigenetic effect or is there is a transcription factor that is just continually activated?

Martin Bushell: We think that it is an epigenetic change but which could be being reinforced by some kind of feedback as well. Now there is some evidence to suggest that the promoter of miR-483 can be independent from IGF2, and that can be epigenetically modified; there is a CPG island in the vicinity that could be participating in that modulation.

Markus Stoffel: So you don't always see co-regulation with IGF2? You think it has its own regulatory site?

Martin Bushell: Some of the promoters do show a slight increase in the low protein offspring, but that isn't sufficient to account for the increase in miR-483. It has been shown that miR-483 has an independent promoter.

Pål Saetrom: It seemed that you had a smaller effect on differentiation when knocking out GDF3 than miR-483. Are there additional targets for miR-483 that could contribute?

Martin Bushell: We are only looking at one target out of many, but one has to be careful when using miR-483 at very high levels because it could overwhelm the system with widespread effects. So I always take those experiments with a bit of a pinch of salt. You are right also that the effects were smaller with GDF3 depletion but we need to be careful in view of the experimental conditions.

Leif Groop What happens in the pancreatic islets? The effects on adipose tissue are interesting but it doesn't really determine whether you get diabetes or not. Do you know how GDF3 is expressed in islets?

Martin Bushell: We don't see miR-483 changing in any other tissues that we have looked at.

Pascal Barbry: We have worked a little with the same miRNA in the context of wound healing in keratinocytes and we found that one important target was the cell division cycle (CDC). So have you looked in your models to see if it was acting on this target and other targets besides GDF3?

Martin Bushell: No - but I am sure it is acting through other targets.

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6. Cross-kingdom regulation by plant microRNA from rice

Chen-Yu Zhang:

I wish to present studies on the cross-kingdom regulation by plant miRNA[15] and illustrate this with the miRNA of rice. We know that the miRNAs have been reported to be involved in almost all of the biological functions across much of biology. However, before 2008 investigators just focused on studying cellular miRNA since it was thought that extra-cellular RNA was unstable. Then around 2007 we identified a circulating stable miRNA in blood that can serve as a class of biomarkers for disease[16]. This concept is now accepted. Since then we have found three types of circulating miRNAs: one relating to on-going disease e.g. cancer as such, another for tissue specificity e.g. for lung rather than stomach cancer and a third miRNA group relating to the immune system response to the disease. We also found that every type of cell can secrete miRNA[17].

Given the remarkable stability of some of these miRNAs I wondered if one could detect miRNAs of plant origin in human blood. We then found a number of plant miRNAs in human serum with some very high copy numbers similar to human derived miRNAs. The sequences

of these miRNAs do not match the human sequences so this was our preliminary evidence for plant miRNAs in human blood. We then showed exogenous miRNAs in human, mouse and horse serum using semi quantitative PCR in fmol/g quantities. The absorption of plant miRNAs through the gut involved a series of analyses. First we assessed the miRNAs in the diet; in the chow diet we fed our mice we had quite high amounts of rice miRNA and this persisted in the cooked rice. On feeding mice with the enriched rice miRNAs the same miRNAs were found in the serum and liver.

After this we synthesised the miRNA in both methylated and unmethylated forms and observed the same miRNAs - whether methylated or not - in the serum and liver after feeding. Testing with pre-miRNA failed to reveal absorption - only mature single stranded miRNA was absorbed. It was also evident on testing under acidic conditions that the mature miRNA could survive the acid conditions of the stomach.

Potential metabolic activity of absorbed plant miRNA in mammalian systems.

We observed that the miRNA 168a which we were studying from the rice miRNA had complementary bases to a sequence in the binding site on exon 4 rather than in the 3' UTR region of the low-density lipoprotein receptor adaptor protein 1(LDLRAP1) in the liver. These complementary sequences of LDLRAP1 are highly conserved across the species being found in humans, primates and rodents. We then found that the miRNA168a inhibits LDLRAP1 in vitro with direct binding of the miRNA to the exon 4 part of the DNA base sequences of the LDLRAP1 gene where it inhibits protein translation and markedly inhibits LDLRAP 1 expression.

To then move into more biological relevant systems we transfected CACO2 cells with the miRNA 168a and harvested the micro vesicles containing the miRNA and then transferred these to Hep G2 liver cells to see whether it had any activity in this cell culture system. The results showed a clear repression of LDL activity in a dose dependent manner. The micro vesicles already have the miRNA 168a associated with Argonaute 2 (Ago2), which is a standard component of the RNA induced silencing complex (RISC). On passage to the Hep G2 cells the miRNA then associates with the recipient cell's own Ago 2 which is already linked to the LDLRAP1 messenger RNA.

Given these cell culture analyses we then needed to see whether this phenomenon could be replicated in physiological terms in vivo. If the LDLRAP1 really is inhibited then the net effect in vivo should be an increase in plasma levels of LDL since the receptor activity is reduced and less available to take up and clear the LDL lipoproteins from the bloodstream. So we undertook a longer term study in mice where we fed them either the chow or rice diets. Within the first day we documented the elevated miRNA 168a in the blood and this increase persisted throughout the week with what seemed to be a progressive fall in LDLRAP1 protein levels. We were surprised that such low levels of exogenous miRNA in the serum and liver could not only shut down the target gene but lead to an increase in plasma LDL levels. If we produce an antagomir to miRNA 168a then we get a partial reversal of the inhibition of LDLRAP1 and a lower blood LDL lipoprotein level,

The antiviral effects of plant miRNAs

So far we have found about 50 exogenous miRNAs in blood. The absorption by the gut involves these miRNAs being transferred into the blood stream in micro vesicles from the intestinal cells. But we still needed to see whether this finding of ours was a chance finding without wider significance or whether there really is a general phenomenon of cross kingdom regulation of metabolism[18]. So let us think more broadly and consider the thousand year old Chinese medical treatment of influenza by giving the patients an extract of the herb Lonicera Japonica which contains the plant miRNA 2911. In practice we have found high levels of miRNAs in plants and when we boil these plants then we find high levels of the miRNAs in the "soup" made from this boiling process. If our team drink this soup from different plants then we detect the corresponding miRNAs in our blood within an hour and indeed in mice we

found the same with the accumulation of the miRNAs in tissues including the lung after some hours. So we have been testing the binding of these plant miRNAs to different influenza strains and find that they bind to the H1N1, the H5N1 and even the current H7N9 strains. Then we have given mice the H1N1 virus and seen a dramatic fall in their body weight whereas the control mice keep putting on weight normally. If, however, we give the mice a soup containing the miRNA 2911 from the Locinera Japonica herb then we markedly limit the impact of the virus on the mice with no weight loss but modest weight gain over the subsequent 11 days. We also find some reduction in the viral titer in the blood of the mice.

We have now gone on to consider the impact of some other plant miRNAs and antagomirs of the miRNA on the replication of the hepatitis B virus. These antagomir RNAs (amiRNAs) e.g. amiRNA 467 we can insert to produce genetically altered plants e.g. lettuce which then produce extra quantities of the amiRNAs which we know can target the hepatitis B virus. Feeding this amiRNA to mice leads to an accumulation of the amiRNA in the liver. So to study the impact of these artificial siRNAs on the hepatitis virus we showed that the fed mice had a reduction in the hepatitis messenger RNA indicating in vivo activity of these types of plant miRNA and other small inhibitory RNAs on viral infections in mammals. We are now in the process of conducting clinical trials.

Preliminary Discussion

Gerhard Aihauld: I am puzzled because we associate rice eating in China and elsewhere with very low LDL levels whereas you are specifying that rice miRNA 168a increases these levels - are we really looking at something of physiological and nutritional significance?

Chen-Yu Zhang: it is a very good question. We may have missed a lot of the physiological roles and it does seem that miRNA 168 targets the LDL receptor adapter protein 1 to increase LDL levels. But remember, from an evolutionary point of view, animals and humans were often deficiency in nutrients so maintaining a certain level of plasma LDL is an important carrier of the cholesterol and plasma lipids needed for new cell wall formation and tissue development.

Ester Nolte-'t Hoen: We know that the extracellular RNA can be either associated with proteins or with vesicles so is the miRNA 168 from the rice that you see in the serum associated with vesicles, or with proteins?

Chen-Yu Zhang: 60% of the miRNA in the serum 168 is in the micro vesicles.

Esther Nolte-'t Hoen: But is this the fraction that is functional or is it the extracellular 168 associated with protein?

Chen-Yu Zhang: In the micro vesicles they have to be associated with Ago2 but not, of course, if they are associated with protein.

Esther Nolte-'t Hoen: But the serum is full of RNAs - how can they survive?

Chen-Yu Zhang: In the serum there are 3 types of miRNAs. One type is in the vesicle: we think of these as actively secreted. Another kind of serum miRNA is released or extruded from the cells - basically the cell doesn't like them. They are not enriching the micro vesicles but maybe binding to the HDL. The third type of serum miRNA are just in the free form. Why they are resistant to RNA digestion, we don't know.

Bo Angelin: The HDL seems a very suitable way of their being transported and indeed protected in the blood with a specific receptor system for removing them. So have you looked further into that?

Chen-Yu Zhang: One example I have is the liver specific miRNA 122 which is released when liver is stimulated by some injury or other and the miRNA then binds to HDL in the circulation and does not go to selected target tissues.

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7. Chemoprotective diets operating through miRNA: carcinogen versus inhibitor actions

Roderick Dashwood

The carcinogen that I am going to be focusing on is a compound called "PhIP" (2-amino-1-methy-6-phenyl-imidazo[4,5-b]pyridine). It has been a very well studied compound and is one of the most widely produced compounds during the cooking of meat and fish and one of the first mutagenic heterocyclic amines to be tested and shown to be a carcinogen in rodents and primates. In rats it is a multi-organ carcinogen producing colon tumours in the males and mammary gland tumours in the females and designated by the US National Toxicology programme as a likely human carcinogen.

In parallel with these studies over many years, there have been chemo-prevention studies using heterocyclic amines as test carcinogens and looking at all manner of chemo-preventive mechanisms[19;20]. There are chemo-preventive actions of chlorophylls and foods containing chlorophylls, for example spinach. Our initial work focused on the so-called blocking mechanisms - that is the ability, for example, of the chlorophyll molecule to form an actual molecular complex with the heterocyclic amine carcinogen and other carcinogens in food such as aflatoxin. Through this molecular complexing the carcinogen uptake from the GI tract can be remarkably reduced with an inhibition of carcinogenesis. In practical terms the carcinogen and an inhibitor may be eaten together e.g. when you eat a hamburger with a lettuce in a hamburger bun. So this analysis raises the question as to whether once exposed to carcinogens one can have any inhibitory effect with plant sources of anti-carcinogens.

Nakagama's group from Japan developed a protocol where PhIP was given in the diet for 2 weeks, followed by a high fat diet for 4 weeks[21]. This sequence was repeated 3 times with a high fat diet continued throughout the study. After one year about 60% of the animals had colon tumours so this preliminary PhIP plus high fat diet treatment was comparable to giving PhIP continuously in the diet. So this saved enormously on carcinogen and study costs. So we took this basic protocol and a high fat diet so that we could incorporate various chemo-preventive agents into our dietary studies. I will use the impact of spinach to illustrate the effects of plant derived chemo protective agents. When freeze - dried baby spinach constituted 10% of the diet the addition of this spinach inhibited at least half the tumours not only in the colon, the primary target organ, but also in other target organs e.g. skin, small intestine, lung, spleen and liver.

Mechanistically we focused initially on the Wnt signalling pathway, which transmits extracellular signals through receptors to the cytoplasm, and on possible changes in ß catenin and on histone markers, looking at HDAC activity globally. But these analyses did not explain our results. Then Mansi Parasramka with experience in miRNA analyses extracted colon tumours and colonic mucosa using a test miRNA array of 679 unique mature miRNAs. First we assessed control animals and defined only a 3-fold change in either direction in miRNA expressions as valuable with the usual statistically significant limits. We found a large number of miRNAs that were either under- or over-expressed in the various treatment groups when compared with the untreated rats. However, when we just fed spinach without any carcinogen it did not modify the miRNAs dramatically in the colonic mucosa whereas our hierarchical cluster analyses and computational modelling showed that the tumours were very different from the untreated, control colonic mucosa. Then when we simply took the top 50% of most highly expressed miRNAs, and then expression ranked them we found in the PhIP induced colon tumours, that three miRNAs i.e. miR-126, miR-145

and miR-21 were over-expressed and the majority of the most abundantly suppressed miRNAs ones were members of the let-7 family of miRNAs. Classified by increasing degrees of suppression we found the suppressed miRNAs to be let-7b, let-7I, let-7d, let-7f, let-7c, miR-98 (another member of the let-7 family), let-7b and let-7a with miR-215 as the most suppressed miRNA when the rats had been exposed to PhIP. So, in general, as has been published in other cancers, we found more miRNAs were down-regulated or decreased in tumours compared with those that were increased; about 5% of the total miRNA array assessed were increased, and about 15-20% had decreased.

So given all these miRNA changes the issue was where they might act. So we conducted Metacore pathway analyses which assess from data bases potential interactions in a huge number of different metabolic pathways; these potential pathways are continually updated. The analyses were displayed in terms of the closeness of the fit for the selected base sequences in the miRNAs and found to fit a seemingly myriad of pathways. We then sought to see where a miRNA might interact with a common signal in several different pathways. What rapidly emerged was the interaction with C-Myc as we found several maps with C-Myc at the centre and the expected interactions with the let-7 family. We know that Myc is a common disregulated target in these tumours and Myc is, of course, one of the targets of Wnt signalling but LIN-28 came up in many different pathways. Dr Baltimore has noted LIN-28 interacting with the let-7 family members and preventing them from being processed to the mature form. So we predicted that LIN-28 was over-expressed in the tumours and would explain why our let-7 family miRNAs were suppressed. As well as LIN-28 and Myc we also found mapping suggesting Oct-3/4 and Sox2 effects. So we went on to try to confirm these inferences through qRT-PCR and found that many of these predicted mRNA targets were indeed altered in tumours. So we were able to confirm a number of these pluri-potency factors as being important players: SOX2, myc, Nonog and Oct3/4 and both A & B Lin28 were highly overexpressed in the tumours. So that is again consistent with the idea that the let7 family members are down regulated. We then confirmed that these effects are seen at the level of protein expression and this is in keeping with the mechanisms involved and the in vivo effects.

The role of low levels of miR-206 and KLF4

What intrigued us about the marvellous work emerging from the recent Nobel awards to Gurdon and Yamanaka on stem cells was the issue of why, given the importance of Krüppel-like factor 4 (KLF4), KLF4 has not emerged at all in our analyses. Does this mean that KLF4 has no role in carcinogenesis when KLF 4 is a known zinc finger protein with critical roles in early development and stem cell biology? However, it seems to have a contradictory role in tumour development : it can promote tumour genesis in some tissues such as breast and skin, whereas in other cases it can suppress carcinogenesis, including in the colon. Loss of KLF4 has been reported in up to 50% of human sporadic colon and rectal cancers as well as in colonic adenomas. An interesting recent report suggests an auto-regulatory feed-back loop between KLF4 and one particular miRNA, miR-206[22]. MiR-206 has been implicated in a number of different cancers, including breast and lung and it has been reported to affect notch signalling, and thereby it impacts proliferation, metastasis and invasion. We had found that miR-206 was the most highly up-regulated miRNA in tumours relative to normal[23] and this is true not only in the PhIP treated animals. But Rob Chapkin used azoxymethane[24] - a completely different carcinogen, operating as an alkylating agent, not a dietary carcinogen also found miR-206 as the most highly increased miRNA. We had missed it because miR-206 is of low abundance. However, this does not mean it is physiologically unimportant. They may be kept low in concentration for a good reason e.g. they could precipitate a cascade effect metabolically as in the blood clotting cascade. On reanalysis we found miRNA-206 was the most highly increased – by a 100 fold in tumours relative to its normal low normal levels. Metacore pathway analysis shows miR-206 is associated with KLf4. In our PhIP induced colon tumours miR-206 is increased greatly in the colon tumours with significant down-regulation of KLf4. We then obtained 21 human primary colon cancers as a

first assessment and matched controls and we now find very low relative expressions of KLf4 and very high levels of miR-206. Experimentally with cell lines we are also able to show that miR-206 induces falls in KLf4 levels whereas with the miR-206 inhibitor, we saw significant increases in KLf4 expression.

So in summary we have first shown that the carcinogen PhIP is linked to miRNA changes with some of the most highly dysregulated miRNAs belonging to the let-7 family. The computational modeling and target validation identified c-Myc and miRNA-binding proteins Lin28A/Lin28B (Lin28) as key players, along with Sox2, Nanog and Oct-3/4. These targets of altered miRNAs have been implicated in colon tumor recurrence and reduced patient survival, in addition to their role as pluripotency factors. Then dietary spinach given after the PhIP has already been administered, suppressed the tumours and this suppression correlated with elevated levels of let-7 members, and partial normalization of c-*myc*, *Sox2*, *Nanog*, *Oct-3/4*, *HmgA2*, *Dnmt3b* and *P53* expression. So the let-7/c-Myc/Lin28 axis is dysregulated in PhIP-induced rat colon carcinogenesis, and tumor suppressive effects of dietary spinach are associated with partial normalization of this pathway.

However, when we also consider relative changes through unbiased screening of 670 odd miRNAs, we identified miR-206 as the most significantly altered miRNA in PhIP induced colon tumours versus normal colonic mucosa[25]. And in a panel of human primary colon cancers we saw an inverse correlation between miR-206 and Klf4. This was supported by both knockdown and over-expression experiments in colon cancer cells where we saw that over-expression of miR-206 increased cell proliferation. With the inhibitor of miR-206 proliferation was inhibited. We therefore need to ensure we focus on low abundant miRNAs as these may prove in metabolic terms to be of exceptional importance

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Preliminary Discussion

Markus Stoffel: Where is miRNA-206 expressed in the epithelium - in all epithelium cells or just in some precursor cells in the crypts where selectively it might have a very high expression? Have you looked at individual cell populations?

Roderick Dashwood: that's a great question. We want to look in the colonic crypts at the stem cell niche as when you give a carcinogen that is geno-toxic it is probably targeting the stem cells and disregulating stem cell factors and spinach may be normalising just these cells.

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8. Epidemiology and translational aspects of miRNA

Curt Harris

MiRNAs have become recognised as evolutionarily highly conserved throughout the plant and animal kingdoms and we learned early in our understanding of miRNAs that they are also differentially expressed in cancers. The seminal observation was made by Carlo Croce[26] in chronic lymphocytic leukaemia in 2003 - that is a decade after the discovery of miRNAs - and now we have well over 10,000 studies published on miRNAs in cancer. We have been particularly interested in miRNAs for diagnosis, prognosis and therapeutic outcome.

So in risk terms we need to consider how to use our understanding of risk in prevention and screening, and in assessing the prognosis of a patient and whether to treat or not given our current knowledge of the early stage cancer with micro metastases. The biology of the tumour will also affect our choice of therapeutic targets. We have been studying germ line variation in the miRNA network, and one of the places that we and other people have looked is the proteins that are involved in the processing of the miRNAs[27]. There are germline mutations and signal nucleotide polymorphisms can be found in these various proteins that

in some cases are related to the risk of the cancer. One can also have signalling nucleotide polymorphisms in the pri-miRNA - that is the very first transcript of the miRNAs. Croce found that there were 3 families that had miR-16 polymorphisms at one particular site which led to a decrease in miR-16 which acts in chronic lymphocytic leukaemia as a tumour suppressor. And then in New Zealand black mice there is also a polymorphism that is very near this site in the mouse miR-16 and these mice have various lymphatic lymphomas and some leukaemias. There are also signalling nucleotide polymorphisms (SNIPS) in pre-miRNAs that can lead to different processing related with an increased risk of oesophageal cancer. There can also be signalling nucleotide polymorphisms in the seed of the miRNA that can actually change the range of target genes and can increase the risk of pancreatic cancer and early onset breast cancer. And then signalling nucleotide polymorphisms in the RNA regulatory regions can also relate to increased risk of lung cancer[28] where this particular SNIP in a KRAS miRNA complementary site was found to be significantly associated with increased risk for non- small cell lung cancer among moderate smokers. This represented a new paradigm for let-7 miRNAs in lung cancer susceptibility.

Other examples of SNIPs in 3'UTRs include the CXCR2 - that is a receptor for IL-8 and IL-6 in particular - which is associated with a reduced risk of lung cancer in European Americans and the Japanese, but not in Afro-Americans. So not all SNIPS lead to an increase in cancer – they can lead to a decreased risk. These ethnic differences also warn us about the need to study these miRNAs in different populations. This particular snip leads to disruption of miRNA-516 binding with an increased CXCR2 signalling in a number of pathways including an interaction with IL-8 where high serum levels in patients are associated with a lower risk. This may be explained by an increased CXCR2 expression signalling through IL-8 with a consequent increased P53 dependent senescence and a reduced risk of developing lung cancer.

Many cancer cells actually produce IL-8 and in some cases it is an autocrine growth factor. So when we are looking at a single factor, one has to be very careful to put it in the proper context and in some ways we need to relate these changes to human cancer studies. In our initial foray into this we found in lung cancers that the miRNA profiles were significantly different between primary lung cancer and corresponding non-cancerous tissue and among different histological types of cancer[29]. The miRNA expression profiles correlated with the survival of patients with lung adenocarcinomas, including those classified as disease stage I. High hsa-mir-155 and low hsa-let-7a-2 expression correlated with poor survival. The relevance of the miRNA expression signature to clinical outcome was confirmed by real-time RT-PCR analysis of precursor miRNAs and cross-validated with an independent set of adenocarcinomas. These results indicate that miRNA expression profiles are diagnostic and prognostic markers of lung cancer. The reduction in the let-7 confirmed the results Takahashi[30] and Frank Slack[31].

We then looked at six major types of human cancer [32] and asked which of the miRNAs were over-expressed in these six major types of cancer? At that point there were only about 280 miRNAs that were known and the one that came up to the top of the list was miR-21. Since then we know that miR-21 is unregulated in 18 major types of human cancer and is a prognostic biomarker in 12 major types. High levels of miR-21 lead to a poor prognosis and most of these studies have been replicated in the same cancer type by multiple labs and also by our group in multiple ethnic groups.

Inflammation and the role of obesity

Now let's turn to inflammation, obesity and nutrition. Chronic inflammation and infection can increase cancer risk; the inflammation may depend on factors which are largely inherited such as hemochromatosis which leads to hepatic iron overload, inflammation and an increase in liver cancer; Crohn's is also partially familial as are ulcerative colitis and familial pancreatitis. But most frequently the infection and chronic inflammation is related to acquired effects due to microorganisms, viruses, bacteria or parasitic infections. However,

frequently the inflammation also depends on chemical, physical and metabolic conditions such as gastric acid reflux with an increased risk of oesophageal cancer in Barrett's patients. Obesity with inflammation is also an issue. Tobacco smoke, at the latest count, I think has 72 carcinogens, but it is also a highly inflammatory agent and I think the two cooperate with each other. Some time ago 18% of human cancers were related to infection[33], but if you include the parasitic factors it is well over 25% as now set out in the new World Cancer Report from IARC[34].

Inflammation and obesity are closely linked. A decade ago it was observed that fat is infiltrated with macrophages and there is activation in feedback loops between the macrophages, preadipocytes and adipocytes with the release of inflammatory-related cytokines and free radicals[35]. They can be the oxygen-based free radicals or nitrogen based free radicals. We are mostly interested in the nitrogen based free radicals, but either one can lead to protein damage and inhibition in some cases of **c**ysteine-dependent **asp**artate-directed prote**ases** (caspases) involved in apoptosis. However, most people have focused on DNA damage and the repair largely by base excision repair that can lead to mutation. There are also indirect effects through epigenetic changes and also through lipid peroxidation: malondialdehyde and 4-hydroxynonenal are very highly electrophilic chemicals produced by lipid peroxidation. Of course, through COX1 and COX2 one can have all the effects that lead to cell proliferation in cancer[36]. And it is interesting that the NOS2, the nitric oxide synthase protein, forms a complex with COX2 and nitrosylates and activates COX2 3-4 fold so there is a direct physical and functional interaction between the nitric oxide pathway and COX2.

Our hypothesis was that inflammation-related genes are associated with cancer diagnosis and prognosis. We looked at 28 different cytokines and asked the question which of those is associated with an increased risk of, for example, colon cancer? We measured the cytokines and miRNAs in both the non-cancerous tissue and in the cancerous tissue[37] and found in the non-cancerous tissues an increase in IL-10 expression, an anti-inflammatory cytokine, associated with nodal metastases in lung cancer. In the tumour tissue itself, several genes were over-expressed and we were particularly interested in IL-23 which increases proliferation of TH17 cells and increases IL-17 which in some cases can act as a tumour progressor.

So from those analyses we developed, by COX modelling an inflammatory risk score and the high inflammatory risk score reflecting primarily the pro-inflammatory cytokines. We found that a high inflammatory risk signified a poor prognosis over 5 years for colon cancer. In these same tumours and controls. We also analysed miR-21 and high levels of miR-21 and they were again associated with a poor prognosis. Then when we considered these two indices together we observed that if both were low there was quite a good prognosis, if either one was high, there was an intermediate prognosis, and if both were high it signified a very poor prognosis. It is important to do different kinds of statistical analysis to assess which covariates are independent of each other because then the combination of these independent factors provides important information. The combination of protein-coding and non-coding gene expression is a more robust prognostic classifier of early stage cancer in several cancers we have studied. With one biomarker group one can readily misclassify some individuals, whereas different markers misclassify others. So if the two are put together you get a much more robust index of prognosis. The same applies to three independent markers.

We found further evidence of the value of this approach in oesophageal cancer[38] and in lung cancer. At a very early stage of lung cancer when the surgeon considers the tumour has been totally removed with no node infiltration then 20-30% of these patients will still die of their cancer within 5 years. In a Japanese cohort and with validation in a USA/Norwegian cohort we found that the combination of miR-21 and four coding genes (XPO1, BRCA1,HLF1a and DLC1) predicted the prognosis of stage I lung **a**denocarcinoma[39]. So these patients evidently had micro-metastases. Now we have looked in the data bases and

have found in over 1000 individuals in 13 different individual cohorts of stage 1 lung cancer that this 4 gene classifier is associated with a poor prognosis. This applies to stage 1, stage 1A and stage 1B lung cancer.

These analyses are valuable because we now know that metastases occur at a very early point in the tumourigenesis process. Each miRNA has hundreds of targets and we needed to know whether any of these targets, verified in laboratory experiments, is associated with human cancer? MiR-21 inhibits a variety of proteins that have important roles in the development of metastases[40]. Earlier studies by Slack suggested that miR-21 is an oncogene associated with the proliferation of lymphomas and that if you turned off miR-21 in the lymphoma model, the tumours melted away[41]. So that is the classical definition of an oncogene addiction. So this raises the question as to whether miR-21 can be used as a therapeutic target. We have now shown that a signature comprising three miRNAs (miR1290, miR196b, and miR135a) enabled the prediction of a chemotherapeutic response (rather than progression-free and overall survival) with high accuracy in both the test and validation cohorts (82.5% and 77.8%) with platinum based doublet chemotherapy[42]

We have also looked at a US and a Hong Kong cohort of colon cancer and found that high levels of miR-21 limit the value of 5 fluorouracil (FU) therapy[43],[44]. Similar responses have been found in a Japanese and a German cohort. And there is some mechanistic rationale for this, but in four different populations now high levels of miR-21 in the tumour are associated with poor response to this type of chemotherapy. The mechanistic rationale is that in the epidermal growth factor receptor EGFR system KRAS is an effector molecule responsible for signal transduction from ligand-bound EGFR to the nucleus and KRAS activity leads to increases in miR-21. David Baltimore and colleagues showed that IL-6, through an increase in STAT3, can lead to an increase in miR-21, and we have shown that genotoxic stress such as hydroxyl radical ionising radiation will also lead to an increase in miR-21

So what is the implication of miRNAs and inflammation in human cancer? There are many groups developing therapy against miR-21 as one of their targets in cancer and we will see if it actually works. But there is a lot of optimism and the next decade will show us whether that optimism is justified.

Preliminary Discussion.

Cutberto Gaza. Can you do these studies on blood or is it just tissue samples one has to use?

Curtis Harris. We can use different strategies and blood sampling is one approach. I much prefer plasma rather than serum because serum, it is a pathological fluid containing primarily miRNAs from platelets and from blood cell. The vast majority of the miRNAs do not come from the tumour but maybe from the endothelial cells.

Pier Paolo Pandolfi We are dealing with something like 32,000 long coding RNAs, so are you incorporating such tools in diagnostic and prognostic schemes? To restrict ourselves to miRNAs seems to me to be limiting.

Curt Harris: Yes, we and other people are looking at link RNAs as an example and quite a few of the link RNAs are associated with diagnosis and the risk of different kinds of cancers. So that would be another class of mechanistically and statistically independent biomarkers to add to the other biomarkers and may give you a more robust prognostic classifier.

General Discussion following the main individual presentations

The interplay of miRNA in metabolic pathways

Ingemar Emberg: David Baltimore noted that he had looked at basically one single pathway and targeted different points in that pathway. Yet we hear from Curtis Harris and others today that the miRNAs are extremely promiscuous so I always get a little bit suspicious when someone selectively targets one pathway and assigns the problem basically to one molecular system. I guess that we have a range of miRNAs some of which are extremely selective and specific and others which are promiscuous. But what is the underlying concept? If they are broad in their targeting, is there some evolutionary programmatic concept in the targeting, or is it a completely random process affecting everywhere in the cell where there is something that could be hit?

Curt Harris: First of all, you don't know what you haven't tested. Secondly, when you have a complex system developing from stem cells into all the various blood cells, that is very challenging, but that is not as challenging as looking at multiple tissues in an animal or human where a miRNA may have completely the opposite effect in one cell type versus another. I am sure that David Baltimore is very aware of that, but it is particularly relevant when one is thinking about disease progression and therapy. Targeting a specific mechanism can lead to side-effects in certain tissues, and developmental effects as well as therapeutic effects.

Markus Stoffel: At this stage of our understanding when you look at genetic models as David Baltimore did, we try to unravel the complexity and identify the pathway that is responsible for the phenotype. So the approach of many investigators is to define the phenotype, at least in genetic systems, and try to define the direct molecular pathway that it is at least partly responsible for the phenotype. But I think you are right: we have to be very cautious not to make it too simple.

Frank Slack: At the time that the Baltimore lab published their original paper it was still considered reasonable to cherry pick the target that looked most closely related to the thing that you were considering. The field has progressed quite a bit since then and there are now excellent physical means to actually get a better sense of what the true, direct targets are. For example, doing miRNA expression analyses is imperative these days. At the time of David Baltimore original paper on this it wasn't even clear that miRNAs affected the levels of the messenger RNAs so it was even harder to test those predictions directly.

Eva van Rooij: I actually think that it is far more complicated. When we first started looking at a direct miRNA-208 target in an unstressed condition we found hardly any genes changed. But then we started looking at miR-208 targets when there was an increasing amount of hypertension stressing the heart. We found some targets were then changed and were dependent on the level of stress. So the more stress there was, the higher the de-repression of those targets. However, when we then stressed the heart with, for example, a vascular occlusion or the high fat diet, other miRNAs were changed. So that means that the signalling pathways that are activated in the cell actually determine the function of a miRNA during a certain disease condition. So a miRNA has a different function under different stress conditions. So when we want to therapeutically use this information in a disease state e.g. heart failure, where you have multiple stresses then choosing the therapy is not easy and will have different effects in different patients.

Martijn Nolte: I think it was very interesting in David Baltimore's talk that he took this knockout and didn't necessarily want to know about the whole network, but rather to understand the impact on the resulting mouse phenotype. So I do agree with Eva's point that the different miRNAs might be differently regulated and to explain a phenotype in the

mouse one needs to know the real executor protein not just factors that increase or decrease a metabolic pathway

Steve Chan: Our lab is actually very interested in looking at the network based effects of multiple miRNA and multiple pathways. One of the reasons that we are doing so is because we now have a growing list of what we think one miRNA can do to one pathway. As Dr. Stoffel pointed out very nicely in his talk, there are now multiple different miRNAs that can affect different pathways that ultimately can lead to diabetes for instance. But we don't know how they interact with one another. So one of the key questions I think in the field is going to be how are we going to address this? Experimentally it is financially prohibitive to undertake a five gene knockout and then to do all the combinatorial pathways to dissect the overall impact. We are trying to do it computationally but this is not easy and I would be interested to hear anyone else's thoughts about how we begin to take the next steps to finding out how these miRNAs can actually perhaps regulate each other and/or intersect into their pathways for real *in vivo*.

Kenneth Chien: Baltimore's work and the speakers this morning have shown convincingly that these miRNAs are incredibly important regulators. But are they modulatory factors which are pivotally important? Mendelian diseases are caused by mutations in transcription factors - are there any Mendelian diseases, particularly developmental that are caused by mutations in miRNAs? If not, does that tell us something? The miRNAs also have some of the same problems therapeutically in terms of how to deliver them, their specificity, and the strengths of their action. If they only have their effects during stress, then that means there has to be an additional signal to allow their modulatory effect.

Pier Paolo Pandolfi: I think that the miRNA field is inflicting problems on itself! If you come from a signalling perspective, or from a transcription factor perspective, you have no problem in accepting that your kinase has 100 targets. We see 100 targets with a transcription factor - so why should this be different in miRNA biology? What did we do when we had complexity at the transcription factor level? We could do two things: study them one at a time and then do combinatorial knockout, or standard high throughput analyses. It is exactly the same thing here. You can take a revolutionary approach and then do a dual or threefold knockout, or you can take a high road and study all the multiple effects. So I think what David Baltimore showed beautifully is first that the miRNA is playing a crucial role and this is important because there are still people saying the miRNAs are doing fine things but are very subtle in their effects. Sometimes they are not subtle at all: they are tremendously important as David Baltimore showed so brilliantly.

The subtlety of miRNA mechanisms

Howard Chang: I thought one part of Baltimore's talk that was very interesting and informative was that he stressed the point that the miRNA genes are transcribed by polymerase 2 and therefore subject to the same kind of regulation as normal protein coding genes. In the example he showed, the miRNA was controlled by the NFkB system and actually ended up feeding back to NFkB itself. In the subsequent talks we have heard a lot of other examples where miRNA levels are changed or elevated in response to diet or other kinds of stresses, though it is not clear what the instigating signalling or transcription factor networks may be. So we really need to get back to the idea of a network of effectors.

Steve Chan: let's take the comparison between transcription factor biology and miRNA. We have taken the computational network approach and have actually been able to identify a lot of networks of genes and signalling pathways that are cooperative regulated by multiple miRNAs. Many students highlight some cases where one miRNA has a drastic effect and we can identify a linear pathway of effect. In many other cases, however, we can really only see subtleties, so it is easy to get disillusioned about the significance of the biology and think that these miRNAs are not very important molecules. We have taken the approach that co-operativity and synergy might actually play a role, so we have taken these computational

approaches, systematically mutated the binding sites of each one of these genes in a pathway that is regulated by sometimes the same miRNA, or sometimes different miRNAs that converge upon the same pathway, and we find a much more dramatic effect - at least *in vitro*. We haven't done it *in vivo* yet comparing these effects with changing just one miRNA alone. So I bring this up only because I feel co-operativity is going to be a major component of how miRNAs work; I think it is different from what transcription factors do and it necessitates us to think less linearly and perhaps less in a reductionist way. We haven't quite figured out how to do that because much of our ways of trying to prove a mechanism of action is with a linear approach, so the idea of how to think differently in order to encompass all of this information in one coordinated network is I think going to be a very important focus in our understanding of miRNA networks in general.

We can't predict the amount of synergy that is there; all we can really predict at this point is the idea that one miRNA may actually regulate multiple points in the same pathway that ultimately lead to a phenotype. So if we use the phenotype as a readout we can then say that if we mutate one of those targets, we get a certain partial effect - maybe 10% - if you mutate multiple targets, you might actually get different effects in that setting.

Miriam Ragle Aure: Dashwood mentioned that the low abundant miRNAs might have important roles as well and I guess that this also calls for the stoichiometry of this to be considered. Otherwise we are just biased by high expression levels .

We also need to recognise the redundancy in the genome. What we see in breast cancer is that different miRNA members of the same family are altered in a complementary pattern across the patient group so it is not only one miRNA, but the whole family which should be considered as a unit.

Cutberto Garza: I am wondering whether in fact when we speak of redundancy we are often in practice speaking of degeneracy, which I often think of as being very synonymous with subtlety? Because if you are looking at a functional outcome, while it is true that we may want to look at a wide network, one of the reasons why we may have subtle quantitative effects is because the system is marked not so much by redundancy, as by degeneracy? Is that true of the miRNA system?

Pier Paolo Pandolfi: Subtlety is additive - if you have a subtle 5% reduction of 100 genes, it can be catastrophic. I think in my mind miRNA and non-coding RNA is forcing us to think in a very different way vis-à-vis having sufficient, or dominant or negative recessive traits that we have been trained to discuss in diseases. We have discussed extensively about why insufficiency happens, and I think we really need to develop quantitative assays that go beyond yes or no or nothing in terms of miRNA. These in diagnostic and in therapy terms, imposes on us a tremendous challenge because we are all used to thinking in stark black or white terms. Curtis Harris has discussed the importance of subtle variation in cancer. From a scientific perspective we have been working in the last 40 years on 2% of the genome. So all of a sudden I realised that I had spent my life working on 2% of the genome, and then there is the 98% which we haven't transcribed. What if this 98% is highly relevant? In cancer therapy we haven't cured cancer by just going after a single kinase, or a single tumour suppressor.

Jørgen Kjems: I think one of the things you have to keep in mind when we are trying to compare MiRNA effects with a transcription effect is that when you turn on a transcription with a factor it takes maybe a whole day to transcribe the gene: it is a very slow process whereas the miRNA has the potential of acting much faster. Even though they are made from a longer transcript, sometimes they can be activated very quickly by either things that inactivate them or activate them. Therefore we have to keep in mind that this regulation is occurring on a different timescale, and therefore we have to investigate them on this timescale and that means that we really have to go down to more single cell analysis because more and more evidence suggests that cells are stochastically expressing genes; they turn on sometimes and go off for hours. So we really have to move down to the cellular

level. And even when you go to the cellular level, we know that it is not just enough to be in the same cell; it has to be at the same time point. Messenger RNAs are usually only there in the cell in a few copies, so when you are expressing some proteins from a cell, it can be 2 or 3 copies of a messenger RNA. That means that a few miRNAs in the right spot would easily do much more than a whole lot in a different place. In that context it is important to remember that there have been several publications now showing that there are bodies in the cell collecting miRNAs and making them inactive so these things are very, very complicated. I think that is why there is so much discrepancy between the different data that we see today. So my point is we should go for single cell analysis in different systems and indeed in cancers too.

Pål Saetrom: I would like to follow up on the single cell analysis and return to the potential function of lowly expressed miRNA because many of the studies that have mapped or identified differentially expressed miRNAs have really looked at cell populations and whether those are heterogeneous or homogeneous is really not that clear in all cases. So you could imagine that if you have an apparently lowly expressed miRNA in the cell population, this miRNA could actually be highly expressed in a particular sub-population and there exert its function. So I would also really like to see more single cell studies.

Martin Bushell: The clip technology should give us a complete idea about the target repertoires and then we can go in and figure out the biological function of those interactions and then predict off-target effects potentially and go from there.

Chen-Yu Zhang: Maybe under physiological conditions, the protein dominantly regulates the physiological condition and miRNA maybe hasß just a 5% to 10% effect but miRNA may be important for non-physiological conditions with secreted as well as cellular miRNA function involved.

Frank Slack: Curt Harris mentioned some of the genetic systems in the mouse that my lab has produced with miRNAs. That work was really influenced by my interest in developmental genetics when I started my miRNA career working on C-elegans. This was the micro-organism where miRNAs were first discovered, so this has dramatically influenced the field. The studies, although informative, have also misled the field to a certain extent.

The very first miRNA ever discovered was lin-4, which is induced by food. Nobody really knows how and we still don't really understand how it gets turned on. The first 3 miRNAs ever discovered, lin-4, let-7 and Isy-6 all work in negative feedback loops with transcription factors. So when they get turned on, they then switch off the transcription factors that turned them on, so I think that is going to be a recurring theme about miRNAs. In those three cases, we know definitively that there is one key target gene because genetic suppressor analyses were done before we knew about computational biology and how you could predict things based on complementarity; people did suppressor screens for suppressor mutations of those particular miRNA mutants and key suppressor genes came out which were later shown to be the direct targets of those miRNAs. So in the case of lin-4, you can completely suppress the phenotype – see some of Victor Ambros's early papers[45] where the mutants are dramatic, and yet if you take out the lin-14 gene then the worms go back to being pretty normal looking. The same is true of let-7; if you take out DAF-12 or hunchback type 1 & 2 transcription factors, the phenotype goes back to almost the wild type. And in the case of Isi-6 if you take out one gene then they again go back to being pretty much normal.

The last point relates to lowly expressed miRNAs. The Isy-6 gene is made in one cell in C-elegans, and yet when you knock out the gene you get a dramatic phenotype of the brain with two cells now having the same function in the brain normally played by only one cell. These early discoveries from c-elegans pointed the way in describing discrete functions for some miRNAs anyway. So I think it is very important to be thinking about the roles for some miRNAs in isolation. The case of miR-375 in islet cells is also a good example of a unique function which was isolated by Stoffel's Lab[46,47].

Long non-coding RNAs

Howard Chang: I would like to expand a little on the fundamental mechanisms and points brought up by David Baltimore and by Esther's and expanded on by Pier Paolo Pandolfi. Long non-coding RNAs are RNAs where there may be 200 nucleotides; sometimes they are arranged into tens of thousands of kilobases. These long non-coding RNAs have been implicated in epigenetic processes by controlling events on chromatin which then allows the transmission of information over subsequent cell generation. Some of the mechanisms include the fact that many RNAs can act as guides to bring a particular protein complex into conjunction with nucleic acids, either DNA or RNA[48, 49,50,51,52] . The link RNA specifies the set of targets, the cargo protein complex specifies the biological outcome, either gene silencing, or gene activation. Other link RNAs can act as scaffolds bringing multiple protein complexes together, so they can actually gather all together for the target gene[53.] Yet additional link RNAs are literally link enhancers[54,55] which target genes to create 3-dimensional chromosomal conformations in gene activation states and, when these regulatory events are done, there are other RNAs that are decoys[56] - they bind to the protein complex, move it away from chromatin and deactivate the gene regulatory process.

An example, particularly relevant to our discussion today, is a long noncoding RNA(lnc-RNA) that has been recently described by my lab[51] and the lab of Paul Khavari[57]. It is very interesting for 2 reasons: one is that it acts in the exact opposite way to miRNAs because the lnc-RNA is required to stabilise the messenger RNA's continuous specific sequence. So the binding of this lnc-RNA to these messenger RNAs selectively stabilises them over time as shown by an actinomycin D pulse -trace experiment. If you knock down this lnc-RNA, the half- life of the messenger RNA is now very, very short. So the lnc-RNA has the exact opposite mechanism as that of a miRNA. Whereas the miRNA turns off target mRNAs , this lnc-RNA actually stabilises them. So a gene may only be expressed if you knockout a miRNA but a knock out of the lnc-RNA means that then the protein cannot be expressed. And really hundreds of other genes in this programme are no longer expressed.

This lnc-RNA is actually itself highly expressed in terminally differentiated skin. So when Esther was talking about breast milk and active nursing of the child, I imagine that this lnc-RNA will be, potentially, one of the most highly abundant non-coding RNA products from the skin transmitted with milk during the act of nursing. But it would be very different if you got your milk in a bottle and there is no skin-to-skin contact.

Hans Krokan: Your study seems to show the loss of staining of the lnc-RNA on the surface of the skin but are you sure that the cells themselves were not just lost?

Howard Chang: No, we know from other experiments that the cells are there; they just maintain their progenitor state and fail to differentiate.

Leif Groop: I was wondering because in the diabetes field we found almost all the genetic variants in the intergenic regions. Quite a few of the diabetes gene variants may actually involve the lnc-RNAs and thereby provide an explanation for diabetes susceptibility. In the presentations today there were quite a few single nucleotide polymorphisms(SNPs) in genes in the target regions for miRNAs action but actually how many SNPs are in the miRNAs genes themselves?

Howard Chang: I don't know much about SNPs in miRNA genes. We recently did a study of messenger RNA secondary structures and found many such variants - SNPs that would change the secondary structure near miRNA target sites so I think that the structural state allows the variants to have important deleterious effects.

Markus Stoffel: I have a question about the lnc-RNAs with their effects opposite to those of miRNAs. Are most lnc-RNAs in comparison to the miRNAs not conserved in evolutionary terms? What are the factors determining the conservation of some of them?

Howard Chang: I think there is a very telling experiment that was published about a year ago. The observation very commonly is that the lnc-RNA's primary sequence in humans, mice and other species looks very different so it is very discouraging for many people as this implies they are not conserved. But what has been shown is that their functions are actually conserved because a lot of functions working through secondary structures involve very short pieces inside these lnc-RNAs. So your computer algorithm is scanning the whole lnc-RNA and you oftentimes miss that signal. The key experiment that was published by the Bartel lab was showing that lnc-RNAs from fish and human can substitute for the function of the lnc-RNA in fish[58], so you can functionally rescue with these very short pieces the function in these animals lacking the lnc-RNAs. Those effects were shown by functional studies but the primary sequences were not very well conserved. So clearly lnc-RNAs only have very small regions that are sequence-conserved for the mediated function.

Curt Harris: In relation to the question about SNPs in miRNAs themselves, there are quite a number of examples and the challenge is those examples is their relation to function. There are examples of that, including miR-16 and miR-146a, mentioned by David Baltimore, where the changes signify alterations in biological function and the risk of disease- in David's case cancer.

Circular RNAs

Jørgen Kjems: I just want to add to this complexity by noting that there was recently discovered quite a big group of molecules that could have quite a significant effect on miRNA function. There were a couple of papers hinting at it a couple of years ago - there were a lot of siRNAs in the cells with thousands being found in human cells. These are circular RNAs (ciRNAs). We detected thousands of well-expressed, stable ciRNAs, often showing tissue/developmental-stage-specific expression[59]. We and others recently uncovered the function of one such ciRNA, ciRS-7 as a circular miR-7 inhibitor, which harbours more than 70 conventional miR-7 binding sites. Expression of ciRS-7 efficiently tethers miR-7, resulting in reduced miR-7 activity and increased levels of miR-7–targeted transcripts. However, in contrast with classical competing endogenous RNAs (ceRNAs), ciRS-7 possesses no accessible termini, rendering itself resistant to miRNA-mediated RNA destabilization.

This circular RNA is a highly conserved structure in all higher mammalian systems. What is also always conserved is that they have a slicing site - there is a miRNA e.g. miR671 that can slice miR-7 off so you have a very quick mechanism of turning on and turning off miRNAs. In neurones this siRNA is the most highly expressed gene so most mechanisms in some parts of the neurones are down-regulated. There is than temporal and spatial control of miR-7 release. We also have another circular RNA that regulates miR-138 and there might be many more to come.

Pier Paolo Pandolfi: I think finding circular RNA clip is a seminal discovery especially as there are at least 10,000 circular RNAs. So perhaps each and every transcribed gene can make a linear and a circular form, and the circular form is extremely sturdy. It is not degraded and therefore can be a competing endogenous RNA species that competes for miRNA. The pervasive nature of this process could be important if these forms are also in breast milk, I wanted to ask Jørgen [Kjems] if you know anything about circular RNA being secreted, because since they are extremely stable and resistant to RNA degradation, they could ultimately be the species that is circulating. We are paying a lot of attention as to how miRNA regulates target genes, but we should in the future pay a lot of attention to how miRNA are regulated and the role of circular RNAs competing endogenous RNAs, lnc RNAs as well as messenger RNA to provide a full picture of this regulatory network about which we know so little so far.

Philip James*:* so to follow that argument, do we know if in breast milk there are particular circular RNAs?

Esther Nolte-'t Hoen: It is definitely not in the literature yet.

Jørgen Kjems: We have looked at a lot of different cancer types and there seems to be a very specific expression pattern for some of these circular RNAs which are clearly also tissue-specific and cancer-specific[60]. But it still has to be seen how many of these are actually working as sponsors of messenger RNA transcription. These ciRNAs are usually nuclear but then when they have a target they go to the cytoplasm and they can nucleate their target miRNA. Also the cleaving miRNA that comes with it are also nuclear, so it is kept away from its functioning site. When we look in neurones you can actually follow the noncoding RNAs out in the dendrites and they seem to be attached to microtubules perhaps as their transport mechanism. So I envision that there is a localisation device in the neurone with identifiers for their location along perhaps a metre length of neurone and you can eventually imagine that these miRNAs are kept inactive and then they can be released at certain places in the cell. So we have to study some of these cells intracellularly at specific sites.

MiRNA interactions

Mikael Rydén: We have just looked at the issue of co-operativity in fat cells and it turns out that the miRNAs work in a more additive than synergistic way, even though you affect different transcription factors in the same transcription regulatory network.

Martin Bushell: We have got a lot of miRNAs that are in clusters and you also have different pathways that are coming in and being regulated so these different pathways add to one another - and I think that is going to prove a major mechanism by which one can actually produce massive phenotypes through miRNA regulation.

Markus Stoffel: There is no doubt that we need computational modelling to look at co-operativity networks and its quantitative aspects. I agree that we can be pretty good at modelling transcription factor networks because there are biologically robust, big effects. But this is not the case with miRNAs. We have been very frustrated so far by this but you can take the most extreme cases of a knockout cell in which a whole family of certain miRNAs has been deleted and then compare it with the wild type with very careful expression analysis and then plug this into your network analysis and ask the question "can I predict the phenotype?" So you take this experimental approach, or you can take the available algorithms - the best algorithms of which include the Rayewski and Battelle algorithms to predict miRNA targets. Yet with the best predictor gene you fail to predict the phenotype. This tells me how little we know. So it would be interesting in the future to get very good modellers together with the experimenters and get the two groups working blindly without initial agreements and then see which models really do predict something. This is going to be very important if you want to think about disease biologies, therapy etc.

Steve Chan: I have some suggestive evidence that mature miRNA in cells can be recycled. So even if you don't have high expression levels it can still become more active because the mature miRNA can be used again. My other concern is that people always use the mouse model, like a knockout mice. But we identified the circulating miRNA expression profile in the mouse and in the human and found that they are totally different. So you use the mice model to study the mechanism but this mechanism does not necessarily apply to people.

Howard Chang: What would be a definitive killer experiment? So, you can knockout dicer which takes out all the miRNAs, and you get embryonic lethality. What if you, however, conditionally take it out in specific tissues at the adult stage? Do you get a phenotype? Has that been done? If the regulation is so subtle, then that would argue that the miRNAs are modulatory and are not going to be great targets for drugs.

Curt Harris: Phil Sharp has made that point many times. Especially in cancer cells, the UTR region of a message gets shorter and shorter, and sometimes you lose a seed which miRNA might bind to. So it is interaction in this case between the biology of a message and the biology of a miRNA.

Steve Chan: I noted that David Baltimore specified that the miRNAs are controlled by Pol2 so they themselves are under regulation and can be induced and this fits the response to stress phenomena. But you know there are haploid embryonic stem cells that have been generated from mouse and these can differentiate and make all tissues so it shows that only one copy of the genes is needed although obviously you have decreased levels of mRNA. The genome-wide whole exon sequencing of people has also shown a lot of haploid genes at multiple loci. So given the array of factors it is going to be very difficult to target drug therapy. There are hardly any transcription factors that are great targets for drug development - probably the steroid hormone family receptor is the only one. So I think it is a challenge but I hope I am wrong!

Eva van Rooij: In the heart they have manipulated the Nkx2-5 and α myosin heavy chain Cre and you get cardiac phenotypes[61],[62]. Yes it has also been done in the adult stage and when they are then deleted, for example, with tamoxifen inducible Cre - you then also get a phenotype which is a dilated cardiomyopathy in the heart.[63]

Markus Stoffel: This is a new evolving field so using different approaches we will learn a lot and be able to predict biological function. 8 nucleotides can confer a lot of specificity. So I don't share your concern. In pancreatic beta cells, if there is an inducible Cre and you delete the miRNAs then these cells undergo de-differentiation and the animals become highly diabetic. There is no doubt about it that in this case there is a collective role of miRNA.

Steve Chan: I am arguing that their effects may be so combinatorial and additive in a minor way - not all of them but many of them - that it may be very difficult to figure out targets for a drug - that is the argument.

Curt Harris: I would like to see many more experiments like the ones that Frank Slack has done looking at oncogene "addiction" in which you induce a tumour, with a particular miRNA - this was miR-21 - and then you turn it off and the tumour goes away. That is a good indication that that miRNA, is important for that tumour. Now that needs to be replicated in other cancer types and eventually in humans, but those findings reflect progress.

Philip James*:* Markus Stoffel presented a whole series of selective effects of different miRNAs on liver metabolism, on brown adipose tissue, on uncoupling protein and highlighted insulin resistance at both an hepatic and peripheral level as well as the need quite often to have a stressed state before the effects of the miRNA become evident.

Eva van Rooij: Every miRNA that we have been studying, or at least a large proportion of them, seem to have a tissue specific function and then a more general effect on metabolism.

Philip James*:* Markus highlights the great specificity of a miRNA but we have also noted the multiplicity of pathways potentially affected by a miRNA. So couldn't you have a very selective effect that we are highlighting, but then you have a general, more modest effect on other processes?

Eva van Rooij: But why would miRNAs have a greater tendency to influence metabolism than other aspects?

Curtis Harris: I actually think that most miRNAs have multiple functions and why shouldn't they have functions in regard to metabolism, apoptosis, senescence by affecting single transfection? I think they all do. What is most interesting about Markus's presentation is that in general people have not identified in a knockout mice a specific phenotype - and he has. Part of the explanation is that you have to look at a very specific cell, or cell area, or cell type to see a specificity of action but in general this has not been done. So I would credit Markus with finding a phenotype with a knock-out which most people have not seen.

Leif Groop: I think it is especially important to distinguish primary from secondary effects in diabetes. An example is diabetic complications with some evidence, for instance, that miRNAs could be involved in diabetic nephropathy. Then there are immediate effects

relating to glucose metabolism and the insulin responses in the pancreatic islets and then you see a few miRNAs that show quite profound changes in expression.

Pier Paolo Pandolfi: I tend to agree with Curtis Harris that miRNAs may exert multiple functions and with respect to saturation vis-à-vis the metabolic function that we are interested in. We are studying miRNAs that target a pathway which is relevant to cancer and apoptosis. If you are looking at cancer, you consider them oncogenic or tumour suppressive. So I think there are many miRNAs that will be relevant to metabolism, but as Curtis said, many of those are also relevant to other functions because I tend to see them as a regulator of pathways and pathways are involved in multiple functions.

Philip James*:* But Curtis is still making the point that if you have a miRNA that acts on the specific cell-type with a particularly unusual effect, then in effect that might be the explanation for the associated phenotypic changes.

MiRNA secretion in *Vesicles*

Esther Nolte-'t Hoen: Bacteria, parasites and even viruses induce cells to release different vesicles from their normal array. But we cannot really pinpoint how much vesicles contribute to the total communication processes between cells. Vesicle generation seems to be widespread and an evolutionarily conserved mechanism of communication but one big problem in the field is that we don't yet have any way of really inhibiting the release of these vesicles from cells so that we can pinpoint how these vesicles contribute to the actual communication process between cells. Mechanisms prior to the release of the vesicles within endosomes as well as the release of these vesicles are so important for cellular function that if you inhibit these processes, you also inhibit other processes in the cell: the cell is no longer the same cell but without the vesicles. There are many groups working on the actual mechanisms behind vesicles release and I expect in the coming two years that this mechanism will be better known and that we can really interfere in this process better than we can now.

Frank Slack. How abundant are these types of vesicles in the blood? Is it easy to identify miRNAs this way? Are there more miRNAs in these vesicles than there are free in the serum, for example?

Chen-Yu Zhang: 60% of the exogenous miRNA-168 is in the micro-vesicles and 40% in the micro-vesicle-free plasma. With endogenous miRNA about 16% is in the micro-vesicle. For the miRNA-128 hepatic specific miRNA, 19% is micro-vesicle free so this is very different.

Pier Paolo Pandolfi: The phosphatase and tensin homolog (PTEN) protein, a very famous tumour suppressor, has been found to be secreted in exosomes[64,65] - but there is still some debate as to the importance of PTEN secretion and tumour suppression and its role in transferring information from one cell to another[2].

Chen-Yu Zhang: We have found that monocytes secrete one miR-150 and are delivering it to the epithelial cells which then show increases *in vivo*. Basically, there is also increased atherosclerosis and tumour induced endogenesis. Right now, I am generating monocyte specific miR-150 knockout mice to see what is going on.

[2] More recently the miRNA control of lactation has been shown to involve MiRNA-486 with miR-486 as a downstream regulator of PTEN that is required for the development of the cow mammary gland. See: Li D, Xie X, Wang J, Bian Y, Li Q, Gao X, Wang C. MiR-486 Regulates Lactation and Targets the PTEN Gene in Cow Mammary Glands. PLoS One. 2015 Mar 4;10(3):e0118284. doi: 10.1371/journal.pone.0118284. eCollection 2015. PubMed PMID: 25738494.

Esther Nolte-'t Hoen: There are so many technological things that we have to overcome in this field. Looking at the percentage of miRNAs or any other RNAs associated with vesicles or any other structures is not such an easy question. For example, the way to isolate the vesicles is usually via differential centrifugation but in the final sedimentation step, which is at 100,000xG in the ultracentrifuge, you will not only sediment vesicles, you will also sediment the protein aggregates that can contain RNA. So the only way to really pinpoint that the miRNAs or any RNAs are associated with vesicles is to perform density gradient experiments with the vesicles floating to the top and the protein-associated RNAs at the bottom. This is so time-consuming that many people don't do the experiment and that is why the field is so hampered by technological issues.

Steve Chan. At least in the plasma, we have done copy number calculations. When we are talking about low or poorly expressed miRNA, copy numbers are going to be extremely valuable because it helps us to understand exactly what we are dealing with. So for an endogenous miRNA such as miR-16, which is probably one of the more highly expressed endogenous miRNAs, in the plasma - we calculated about 140,000 copies per microliter in human plasma. So the idea of being able to differentiate between vesicles and extracellular miRNA that have perhaps been packaged in proteins is a really good one. We do have an example of a miRNA that is hypoxy-induced, but yet secreted as a protein complex independent of micro-vesicles. We were able to fractionate out the exozomes and found that our miRNA was not in the exosomes but actually secreted into the serum of persons who were either in a physiological or pathophysiologic hypoxic state. We found that this miRNA, miR-210 is complexed with argo-2. I think it is both a secretion useful for signalling and just a way of getting rid of it from the cell. We found that prolyl-hydroxylation of argo-2 actually effects the secretion of this miRNA in hypoxia and normal oxygen states. So in hypoxia when argo-2 is hydroxylated - at least at the proline residue - we found that hydroxylation actually increases the intracellular retention of this miRNA and prevents subsequent release into the extracellular space. However, upon re-oxygenation, when argo-2 is not prolyl-hydroxylated we were able to find that miR-210 is preferentially released into the extracellular space. So that is one indication that at least we have some degree of regulation of how these might be functioning, especially in the hypoxic situation.

In addition, in *in vitro* studies, using miR-210 knockout recipient cells with extracellular miR-210 placed on top of the cells, the miRNA was able to enter the cells and target gene repression, but the expression levels were not that high. However, the question is whether in normal cell types that have normal amounts of miR-210, does exogenous miR-210 get in and actually change function? The problem is that miR-21 is so highly expressed endogenously throughout the body that we know that the amount entering cells would be of dubious significance unless there was something special about that delivered miRNA. By copy numbers alone, I don't think that there would necessarily be a great biological effect.

Kendal Herschi: My concern is that the technology to isolate these MiRNAs in systems such as blood is so different from lab to lab that one wonders whether it is really a biomarker? When I try to reproduce the result myself, I get something very different from the lab that did it before. Is it because I did a spin at a different speed from someone else? So there really does have to be some standardisation in the sera isolation sequencing because I can't produce what someone else is doing.

Kenneth Chien: it is almost a developmental biology problem. Actually the tools exist, I think, to address this. So there are knockout embryonic stem cells and you clearly could make them homozygous deficient or derive them from animals that have completely lost the miRNA and choose one where you have a very well defined target for miRNA which is also sensitive to being turned on. Then you make chimeras and essentially undertake a blastocyst injection and see whether or not that deficient cell will then turn on the reporter gene. Or even better an endogenous gene or an endogenous function. If it can't do that in a cell non-autonomous manner then I think that the chances are extremely low that it is

operative. Now you could say another miRNA might do it, but I think that would be very challenging. The thing I like about the worm analysis is that it is a simple genetic organism.

Jørgen Kjems: We seem to assume that the miRNAs are targeting other cells, but in many ways one could also imagine that this is a way for the cell to get rid of miRNAs. The problem with miRNAs is that they have a very long lifespan, so if you measure them the lifespan is days or even a week so if the cell needs to get rid of them, it could be a very good way of just exporting them out of the cells in this way. It has also been known for many years that in cells that are undergoing stress, there are endogenous retroviruses which form bio-particles inside so perhaps these endogenous viruses are somehow producing some of these particles, and just by coincidence are assembling miRNAs as well.

Robert Chapkin. For someone like myself who has made a career working with lipid vesicles I concur with Esther: there is a failure to standardise methodology but there are more papers coming out now that are clearly addressing that point. I am not disparaging the great work done up to now, but floating membranes are incredibly complex and doing it uniformly - even in one lab, let alone across labs is not a trivial challenge. You mentioned that dendritic cells don't shed these vesicles when they are in a quiescent state, but when they become stimulated, they all of a sudden gear up and release these vesicles in order to influence, in this case, a T-cell population. So what mechanistically do you think is going on there to turn on this vesicular transport to the plasma membrane and then the release of these vesicles? Do you know any specific signals in the dendritic cells that are regulating that, or is it a big black box currently?

Esther Nolte-'t Hoen: In the quiescent state, there is some vesicle release but far less than when you activate the dendritic cells. It is both the number of vesicles that are released that is increased, after activation, but also the composition of the vesicles. For example because we have a single vesicle analysis method, we can see that the amount of miRNA per vesicle is actually increasing when the dendritic cell is activated. If you look at the exozomes as the vesicle sub-population, they arise from the multi-vesicular bodies in the dendritic cells and we have seen that there is diversity in the multi-vesicular bodies. Some multi-vesicular bodies fuse with the lysosomes and then the confluence of the multi-vesicular bodies is degraded. But apparently there is another class of multi-vesicle bodies that is not fusing with the lysosomes but is fusing with the plasma membrane and then these inter-luminal vesicles are released as exozomes. So it may be that a routing or the origin of the multi-vesicular bodies is changed when the dendritic cell is activated. And then there is relatively more release by fusion of the multi-vesicle bodies with the plasma membrane.

Markus Stoffel: I agree that there is a lot of variability between labs in measurement. We tried to establish the levels of a miRNA which is very specifically expressed in one organ like miR-122 and tried to find its concentration in other tissues. We didn't just use our own data, but also publicly available data. And the problem that I had with possible remote signalling is that you can find miRNA in other tissues, but the levels are extremely low. It is so low that I think we can say with some certainty that they don't have a biological role. This is also true for the rice miRNA, which is present in many plants, in that one can find it absorbed but at such low concentrations that I do not think it is active. (See below: section from page 42). So my problem with the concept of remote signalling is that the low concentrations observed remotely are not compatible with physiological function.

Chen-Yu Zhang: MiRNA is very selectively packaged and then secreted when cells get specific signals. The selective packaging is essential for the specificity and the specificity is also essential for their function. We don't know what the mechanism for the selective packaging is. The second issue is the regulatory mechanism for the exozome or cellular vesicle secretion. It seems to be similar to insulin granule secretion. The other point relates to low observed levels of miRNA in other tissues. Normally, if the recipient cells don't have elevated levels of pre-miRNA, we think that this is exogenous miRNA i.e. secreted miRNA from other tissues.

Markus Stoffel: there is no doubt miRNA is secreted but I still haven't seen the evidence that you get enough uptake of miRNA from a remote site by a cell which does not have this as an endogenous miRNA and then find a biological effect.

Chen-Yu Zhang: OK, two years ago we published a paper that we calculated that per recipient cell, at least, 300 copies per cell were taken up and we know that 200 copies of the cell is functional with endogenous miRNA.[66] It is important to use a deep-sequencing technique to screen the circulating exogenous miRNAs. There are thousands of miRNAs in plants. However, only 30 known plant miRNAs were detected in human plasma. Thus, the abundance of miRNAs in plant may not accurately reflect the distribution of miRNAs in animal tissues. Therefore, the authors, selecting miRNAs based on their high levels in the plant, might detect inappropriate miRNAs. Secondly an internal control or reference gene should be employed in the qRT-PCR assay to normalize miRNAs in plasma. Proper normalization is critical for quantitative analysis of extracellular miRNAs, as variations in the amount of material, sample collection, RNA extraction and enzymatic efficiency can introduce potential bias and contribute to quantification errors

Markus Stoffel: We did this with lots of RNAs and we took all the specific miRNAS but maybe we didn't look at the right ones. I think the killer experiments using very clean genetics, are extremely important and we need to solve that question.

MiRNAs from an evolutionary perspective

Ingemar Emberg: I learned a lot from Esther's wonderful introduction and I remember she showed these pie diagrams showing an increasing number of miRNA as one moved from bacteria, to yeast, then to higher organisms and then to man - there was an explosion of the range of miRNAs going from unicellular to multicellular organisms. I think it is much more likely that MiRNAs are involved in network modulation and fine-tuning and adding robustness to the intracellular network in some way. Maybe the metabolic effects are just a reflection of the general intracellular networks which are modulated by miRNAs in a very complex and difficult way to analyse today with the tools that we have. I know another system, tyrosine kinases, that have exploded in number as one goes from - a very primitive ancestor of one tyrosine kinase in yeast through to multicellular animals with hundreds of tyrosine kinases.

Esther Nolte-'t Hoen: It is not so much the percentage of miRNAs that have exploded but the total percentage of non-coding regions that were transcribed. So what exactly the graph would look like when you would have only miRNAs, I am not too sure. I am sure that they increase, but in what percentage they increase, I am not too sure.

Patrick Stover: I think for me the whole miRNA story is yet another example of why there are so few penetrant polymorphisms in metabolic enzymes - the system is so heavily buffered. We had a slew of literature about 10 years ago showing how nutrients regulate translation which gives you more protein, which overcomes any potential effect of a single nucleotide polymorphism on flux through a system. Now we have another layer of regulation demonstrating again how you can regulate these networks that affect metabolism and buffer it. What was most impressive to me was the way that miR-7 knockout, or rather its over-expression actually gives you a MODY diabetic phenotype which is so rare in these systems - to be able to get through the complex buffering systems to actually get to a different phenotype.

Steve Chan: I just want to come back to the idea about whether metabolism from an evolutionary standpoint really is that important and whether miRNA should be regulating it. We have taken a separate type of approach: we are not necessarily looking at glucose metabolism *per se*, but we have actually been very interested in mitochondrial metabolism such as the electron transport chain, respiratory complexes and so forth. From that standpoint, more than a century ago Louis Pasteur first described the switch which we know partially as the Marburg-phenotype - it is also called the Pasteur effect when it is occurring in

hypoxic states where you can actually switch from mitochondrial metabolism and oxidative phosphorylation to a glycolytic phenotype. This happens in yeast all the way up to mammalian cells and it is very important for the survival of cells in the acute state whereas it is detrimental in the chronic state. So we have been very interested in that from an evolutionary standpoint. So if you take a step back and you look at all the 3' untranslated regions(UTRs) of the mitochondrial respiratory complexes, you find that those 3 'UTRs for the most part are very short, if there at all, and maybe 50-100 nucleotides in length. Most of the miRNA target algorithms don't predict miRNA to be regulating these particular complexes, so it has been proposed that perhaps these are constitutively expressed and always very important for function. We have actually found that there are certain hypoxia-relevant miRNA that can affect the activity of those complexes, but operate through the metal bio-prosthetic groups that are actually important in those electron transport chains which are not represented by the transcriptome or the proteome. So I guess ultimately I would say that from an evolutionary perspective, that miRNA still are very important even in the mitochondria and protein activities with specific targets are affected by miRNAs only indirectly. We are probably going to find a lot more miRNA that affect metabolic phenotypes in very important ways but through indirect actions on other aspects of these complex pathways.

Frank Slack: As far as I can tell, there is only one known single-celled organism that is known to have miRNAs and that is Chlamydomonas which is a single-celled alga. No fungi, no bacteria, have miRNAs; they might have functional RNAs that have a similar role, but they are not miRNAs. Up until just a few years ago most of us considered that miRNAs evolved in multicellular organisms and that they evolved in order to allow for specialisation of cells during the multicellularity of their life. So, if you just take that point of view, one might consider that metabolism is so essential for all life, including single-cell organisms. So, given the absence of miRNAs in single-celled organisms, miRNAs probably didn't evolve necessarily to regulate metabolism, *per se*. However, that doesn't mean that over evolutionary time they did not evolve to have functions regulating metabolism. I would completely concur with the discussion today that most miRNAs when knocked-out, for example, in c-elegans, do not result in an observable phenotype, but when you stress the animals, then you see the different phenotypes emerging. So I guess my point is that they didn't evolve necessarily to regulate core aspects of metabolism, but perhaps to regulate networks of metabolic genes and to cope with the capacity to adapt to circumstances.

Patrick Stover: But if you look at rates of gene evolution, where you have some sort of mutation that provides a positive advantage, then its prevalence expands in the population due to selective pressures. These genes are mostly enriched in regulatory metabolic processes relating to nutrients and immunity. There is no other class of genes in a system other than metabolism and immunity that show such positive selection, rapid evolution and adaptation. Is it not in their role in immunity and metabolism that we see most of the miRNA story?

Howard Chang One class of non-coding RNAs that has not been discussed so far are the riboswitches which are very important in bacteria. These are non-coding RNAs that are directly appended to the mRNAs encoding biosynthetic enzymes and the riboswitches directly sense the product levels of the biosynthetic pathways and therefore regulate beta translation that is already sometimes the termination of those transcripts. So that is a very prevalent mechanism of regulation metabolism in prokaryote and to my knowledge is largely lacking in eukaryotes - in multicellular organisms. We now have a large number of miRNAs and perhaps other kinds of link RNAs. I tend to be more in the Frank Slack's camp in thinking that miRNAs, are involved in gene regulation, in coordinating the cooperation or competition among cells and so it is a mechanistic control and fine tune gene regulation. So they will be involved in really many kinds of biological processes, including metabolic regulation. We actually found a lot of non-coding RNAs in evolving complex organs e.g. in the brain which is, metabolically, a very active organ. But they also have a lot to do in cell-

type specification, and when one considers the complex nature of their lineage and also their role in cell-cell interactions we need to recognise the range and importance of non-coding RNAs.

Paul Wilmes: Small RNAs in bacteria are very commonplace; in the world's oceans, for example, it has been shown that there are vast amounts of extracellular small RNAs and they have an intricate secondary structure which provides them with great stability in these environments. Mitochondria, of course, are direct evolutionary descendants of alpha proteobacteria; so one has to think that its regulation may in fact be using small RNAs more akin to those of prokaryotes rather than eukaryotes.

Chen-Yu Zhang: From an evolutionary point of view we should not mix up two separate issues. We are always talking about a protein's function when considering what the miRNA, or non-coding RNA does. However, we should be thinking about the vast area of the genome without coding sequences and these may relate to every type of pathophysiology, to metabolism, to apoptosis etc.

Gerhard Aihauld: The evolutionary importance of the emerging large proportion of non-coding RNAs may not just be involved in developing and maintaining metabolism but also the evolution of temperature regulation. So the difference between poikilothermic and homoeothermic states is marked and of immense importance. So there are very efficient miRNAs involved in brown fat tissue formation and function..

Cutberto Garza: Markus Stoffel made the point I think that there was very little evidence for effective intercellular communication or mechanisms for miRNAs exerting their influence in that way. But that doesn't make sense if what Frank Stack says is correct: if miRNAs evolved in a manner to cope with multicellularity, then either they exert their influences completely indirectly, through metabolic mechanisms that are primarily intracellular, or there has to be some mechanism, either vesicular or some other, for intercellular communication. Otherwise it doesn't make any evolutionary sense.

Ingemar Emberg: When we assess the evolutionary significance of miRNAs we should remember, of course, that cells don't suddenly recognise that they now have a good tool to regulate metabolism! Once the cell has developed a new evolutionary feature like miRNAs, they will use it for anything where it works optimally. One of the successes of this meeting is that we brought miRNAs into the arena of cell communication via vesicles. However, the RNA world is at least as multi-functional as the protein world.

Martin Bushell: Actually we know a very good example of a molecule that is really central to the cell biology - the ribosome - and that is incredibly well conserved, particularly its catalytic core. I was just wondering about the long non-coding RNAs and whether or not - as from what I can see, they have very short elements conserved within the structure so are these RNAs able to carry out huge complex catalytic activity?

Mikael Rydén: I don't know much about prokaryotic organisms but why wouldn't the bacteria communicate with each other through small RNA molecules, whether they are called miRNAs or not. That would be a feasible mechanism of interacting if they are growing in a certain environment then are subjected to differences in pH or whatever - so why wouldn't they be able to communicate with each other?

Ingemar Emberg When considering the prokaryote world and the early evolving chemistries, the early cells, and the early RNAs world then we see different systems taking advantage of newly available systems. In modern biology we see the chloroplast and the mitochondria as examples but if you look at the original metabolic chemistry the process of acquiring different systems from different local environments seems beneficial from an evolutionary point of view. The bacteria show that horizontal gene transfer is quite common although it often fails to embed within the new host and as we became more sophisticated greater barriers to information transfer developed.

Chen-Yu Zhang: We now have the data showing that the bacteria also can secrete outer membrane vesicles (OMVs) similar to the micro vesicles. They contain a lot of small RNA. Basically they secrete small RNAs into the host cells and the host cell accesses the small miRNA and this miRNA then can induce nitric oxide synthase. Bacteria also can communicate with each other via OMVs.

Pål Saetrom: The big issue in taking the short double stranded RNAs (SiRNAs) to the clinic and getting the SiRNAs into the correct cell and this must operate in terms of the micro-vesicles as well. You have to have the right signalling particle or whatever on the vesicle to get it into the correct cell.

Philip James*:* And the receptor in the cell to pick it up?

Pål Saetrom: Yes.

Cutberto Garza: I agree one can have communication by direct microvesicle uptake but, of course, the metabolites of miRNA controlled metabolism can be another form of communication. I don't know whether we can distinguish between these two, or possibly other mechanisms but it is clear to me that they must serve a primary signalling role if they evolved later than the unicellular organism.

Jørgen Kjems: When going from a very primitive, single cell organism up to a multicellular organism the genes become much longer, and then include many introns not coding for amino acids. Then differential splicing emerges as a very important specialisation mechanism which is part of the higher mammalian systems. But the polymerases have still the same speed so that means that you cannot regulate anything more on the transcriptional level - that is the disadvantage for the humans, for instance. On average the time for making a gene is about two to three hours, and sometimes it can take a day to make just one transcription. So that means that you cannot regulate metabolism by transcription in these cases. So even with a constant body temperature we still have to eat and turn on metabolism. Since this cannot be done by transcription it has to be done by miRNA and kinases and some of these very quick-acting methods. That is a completely different perspective relating to the roles of miRNAs.

Pier Paolo Pandolfi: Metabolism is a multi-organ function, so organs communicate in many ways and we know by now that non-coding RNAs are very tissue-specific. So my view is that miRNA, long non-coding RNAs, may regulate tissues-specific functions that allow organs to communicate. This can be done very fast because again we are regulating translation, not transcription. One example of such a need for speed is for brain function. The neurones are extremely long making it difficult to transfer the information from the nucleus to the synaptic edge but this can be done very efficiently using non-coding RNAs. So my way of reconciling the fact that miRNAs are integrating function as well as transferring information is that they are a tissue-specific regulator of cross-organ communications. Hence, very relevant for metabolism, but why do we have to see them as regulators of protein? They regulate each and every function and in cancer, you see that many of them are regulating proliferation and survival. Proliferation and survival are also metabolic functions. So I don't really think that they have evolved to regulate metabolism, but certainly they regulate cross-organ, cross-cellular communication in a very specialised way.

Curt Harris: I think that our understanding of miRNAs is evolving and 5 years from now we will look back at our conversation today and say it was really primitive! There are multiple other classes of non-coding RNA on which we are starting to get some data such as the link RNAs that we discussed, But this is a new world for most scientists in their understanding of what the non-coding RNAs' functions are. We will learn a lot from bacteria and single cell organisms, but I agree that the complexity in dealing with multi-interactions between various tissues is going to be key in lots of diseases.

Chen-Yu Zhang: MiRNA are horizontal transferred and mediates co-evolution. So take the bee society with all its females: the Queen and the worker bee are female. The difference is

that when the bees are born, some are fed Royal Jelly while the others are just fed honey that then programmes worker bee development. We noted that from an evolutionary perspective it also appears reasonable to assume that the Queen is closer to the normal insect female, and that the production of a specialized, sterile worker must be a highly costly and very risky strategy requiring tight regulatory control. These observations all seem to imply that it is the worker program that needs to be actively switched on, and, thus, that it is the prospective worker larva that must receive a specific environmental signal (nutritional or other) to activate this program. We then found that the honey contains greater amounts of miRNAs than those found in Royal Jelly that determines Queen bee development[67]. We also found that the feed of developing worker bees contained many miRNAs not found in Royal Jelly. Honey contains plant miRNA that targets certain miRNAs and when supplied to the larval feed of prospective queens they are capable of altering specific adult morphological characters in the direction of the worker bee. One miRNA, miR-184, had a particular effect in altering the physical characteristics of the developing larvae and was also reflected in substantial changes to the larval mRNA expression pattern. These larvae fed differentially do not then develop their ovaries. We also know that the honeybee is recognized for its advanced mental faculties but the worker and Queen bee types are behaviourally very different. So it is interesting to find that the most abundant miRNAs in the worker jelly collectively regulate a number of mRNAs related to different features of brain development.

Philip James: this is completely fascinating and relevant to the present concern about bee viability.

Howard Chang: I would like to take us back to fundamental principles, thinking about why cells use RNA for different kinds of regulation. RNA has a primary sequence, that is in itself is a form of information. Then the RNA can base-pair with itself and also with other molecules, to form complex secondary and tertiary structures. So that means that you can have both the information and potentially functional output from the same molecule. So one really nice idea that is an example that relates to Gerhard's point about temperature is that a very fundamental mechanism controlling temperature in bacteria is the presence of RNA thermometers. There is a very simple property of hybridisation in base pairing and it seems to directly sense temperature. And then when that unwinds, the proteins get made and that is the heat shock response in bacteria. We last year[68] published a study looking for RNA thermometers in eukaryotes and chose yeast - actually thousands of such thermometers also exist in yeast. These messengers basically have a different function in yeast and predict the pattern of RNA decay in vivo during heat shock. The exosome complex recognizes unpaired bases during heat shock to degrade these RNAs, coupling intrinsic structural stabilities to gene regulation. Thus, genome-wide structural dynamics of RNA can parse functional elements of the transcriptome and reveal diverse biological insights so it is a very prevalent mode of regulation.

I think a very interesting idea is that these RNA based mechanisms compete with each other and some of these small RNAs exert transposon control. Other classes of small RNA have been identified as silencing RNAs, including piwi-interacting RNA (piRNA) and its subspecies repeat associated small interfering RNA (rasiRNA). So we can think of miRNA and miRNA seeds as another version of repetitive information. You have little sequences in the genome that are essentially the same and they have some sort of competition - the miRNA will shut down the messenger RNA, and the same kind of idea may exist for let's say link RNAs. These basically relate to the sequences and silence them, in this case through epigenetic mechanisms. The sequence composition in link RNAs have small regions that have high structural capacity and are often repeated and often linked by intervening sequences that are very flexible. So unlike messenger RNAs where there are very strict rules for the frame of the sequence and for their translation the sequence appears to be much more flexible for long encoding RNAs. So I am setting out there the concept of evolution based on different niches with information competing for the same niche and hence providing the basis for miRNA silencing messenger RNA.

Roderick Dashwood: The question came up as to why do we see evolution of the miRNAs through to humans, whereas you don't see that with the link RNAs. I think you gave a great answer that it is the little particular piece of the link RNA acting as part of the whole tertiary structure. And we saw nice diagrams about how the tertiary structure can give rise, as it were, to a scaffold for bringing in different proteins in other contexts. I think we are also going to see cross-talk between different types of non- coding RNAs and an example is Cep-1, a colon cancer associated transcript 1, a link RNA which has both different lengths of transcripts that do different things and there is also within those miRNA elements that are regulated as well. And depending on conditions such as oxidative stress or genotoxic stress for example in colon cancer cells, you can shift the balance. So you may have a tertiary structure of a link RNA available for docking with a protein but where a miRNA might come in and also bind there preventing access to the recognition site. So we are going to be having cross-talk between different types of non-coding RNAs.

Pier Paolo Pandolfi: we have put forward this idea of competition and cross-talk among RNA species[69] calling these competing endogenous RNAs. We postulated that 10 RNA species in these species can cross-talk when appropriate concentrations and localisation requirements are fulfilled. And we think that in cancer you have a profound filtration among species, with a shortening of the 3' UTRs. So these massive transcripts could create aberrant cross-talk among RNAs. And what we find extremely appealing is that we can predict such interactions simply looking, for instance, at miRNA responsive elements because you can really read the information and predict which RNA would talk with which RNA. We postulated that this would apply to any RNA species and now through this new technology of CLASH[70] it has been found that miRNA can bind ki RNA, and sno RNA can bind ki RNA. So there is a pervasive RNA cross-talk which again brings us back to our original question: do RNA have their own independent way to communicate and the answer is probably yes. So again the cross-regulated chatter impinges on the protein dimension. If you come from an RNA perspective, you are saying that this is the factor whereby RNAs can talk to each other.

Breast Milk miRNA

Esther Nolte-'t Hoen: We do see a lot of keratin in very clean samples of breast milk so I think there are definitely epithelial cells that are transferred from the mother to the child and I think it is a really interesting idea to look at the lnc RNAs as well. Some of the small non-coding RNAs are known to function as decoys for certain RNAs. For example, the small RNAs derived from the untranslated region may also be able to bind to certain proteins and act as decoys for the other regulating RNAs. There are also small non-coding RNAs which serve as guides for other RNAs to act. So I think that within the near future we will look far beyond the miRNAs and look at the complex regulatory processes.

Chen-Yu Zhang: We also identified miRNA in the breast milk and in cow's milk there is a lot of species differences. And colostrum and mature milks also differ in their miRNA expression profile. Interestingly, if you think the miRNA in the milk is also important for human health and physiology, that is interesting because all the milks we have studied are enriched with mir-21I but I don't know what does that means. It may be for the infant's development but in adults we might expect it to promote tumour growth. So you have to identify the different types of miRNA in the different sorts of milk.

Esther Nolte-'t Hoen: I think we need to be careful about just looking at the presence of something - it all depends on what happens to these RNAs and whether it enters specific cells and whether the vesicles containing miRNA target some cells under particular conditions.

MiRNAs and the microbiome

Paul Wilmes: We are interested in taking a systems level approach to the analysis of microbial consortia, including those that inhabit the human gastrointestinal tract. You probably know the huge excess of microbial numbers in the human gut compared with the number of cells in the body. There is also the concept that there can be an imbalance in the microbial ecology of the gastrointestinal tract potentially causally linked to a range of different diseases. So we wanted to take proper systematic measurements of microbial consortia using high-resolution molecular tools that are now available[71]. Given the heterogeneous array of microbes you have to take single, unique samples, then extract all of the different biomolecules from this single unique sample and subject it to high resolution OMIC analyses with the resulting data sets then being meaningfully integrated[72].

One can obtain electrophoresis analyses which highlight the differences in microbial community samples when we extract total RNA using our method which is applicable to a range of different communities. We were struck by the significant amount of small RNA in human faecal samples. We rapidly then gained collaborators and David Galas' s group in Seattle then amassed a lot of RNA data from human plasma[73]. When we extended the databases for checking the potential origins of these RNAs these could be traced back to exogenous sources. In mapping these data against a range of different profiles typically found in the human microbiome, we find a very good representation of the RNAs from eukaryotes which are the methanogens in the gastrointestinal tract. We have then applied our systematic approach to analysing the faeces and blood from 10,000 individuals. We have done small RNA sequences and total RNA sequences as well as DNA sequencing on the extracts obtained from both faeces and plasma. We have also spent a considerable amount of diligence and effort on devising a bioinformatic pipeline that allows us to really discern different sequences. The plasma spectrum, of course, contains a significant amount of what we here term vertebrate small RNAs because some of the sequences e.g. in miR-486 are identical in human and bovine samples whereas other isomers are clearly bovine. But what we also see is a significant amount of plant small RNAs. Fungal RNAs are very prominent and we hypothesise that these are diet-derived because we see a very strong signature for fungi that are typically associated with food contamination. Then the bacterial representation is primarily derived from the human gastrointestinal tract. We can also see really quite distinct differences between individuals. Thus, for example, we found the plant small RNA contingent is much more pronounced compared to the others in the one vegetarian we studied in a small cohort.

Olle Hernell: Have you had a chance to have a look at the microbiome in the small intestine to see how that relates?

Paul Wilmes: This is inherently more difficult so we haven't done that yet, but our data suggest that most of the absorption is actually taking place in the small intestine rather than from the descending colon.

Philip James*:* You said that fungal material you think came from contaminated food - is that right?

Paul Wilmes: The species which we can map most of in the small RNAs in human plasma is in fact from neurospora crassa, that is bread mould - we ingest a lot more fungal biomass than one would think. You only throw bread out once the fungal growth is really rampant but I assume that the bread is probably well colonised by fungi before it is obviously mouldy. We also find good representation of the aspergillus species with a lot of individual specific variation which we explain by the variation in exposure to the built environment.

Kendal Herschi: Wow! Are you using a different technology from others? Because I have looked through some of these databases myself for these things and I don't see it.

Paul Wilmes: I also just heard about the trizol isolation and so on - this is not what we do. You are welcome to look at our method for isolation - it is basically just illumina sequencing

as many people use. I find it surprising that there seems to be no consensus on the analytical approach. The first of our data sets are out there - in fact from our paper late last year so you are welcome to look at these data.

***Philip James**:* but you are doing computational analysis of these putative molecules that you are finding in plasma to work out their microbial type and origin?

Paul Wilmes: Yes, we have spent a lot of time on this because, of course, these are short reads and the potential for cross-mapping is quite significant. So the data that I use is based on information taken from multiple locations in order for us to even consider this as a positive.

Pier Paolo Pandolfi: Could this be due to the fact that macrophages are eating bugs and then they are releasing these into the blood? Are you considering the possibility that in macrophages you have plenty of bugs, small RNAs, simply because they are phagocytosing these bugs and so maybe the difference is not in how you sequence or how you purify RNA, but how clean, or dirty, or how many macrophages you are keeping in the plasma? So some of us here are studying macrophages in the blood and others are studying the output of dendritic cells which may have been taken up by macrophages?

Cutberto Garza: Back in the days when germ-free animals were being studied much more intensively than today there were all sorts of physiological effects that no one could explain, like very small hearts! I am wondering whether anyone is beginning to look at whether there are any linkages between some of the observations that are being made and between small RNA molecules and the physiological effects in the absence of a microbiome.

Paul Wilmes: In a paper published late last year several synthetic, double-stranded mature microRNA-like molecules selected from observed exogenous miRNA sequences and some highly abundant exogenous sequences (bacterial rRNAs) that have the potential to form pre-miRNA-like secondary structures were transfected. These were inserted into a mouse, dicer-deficient, fibroblast cell line[74]. Because of the lack of the dicer protein the dicer deficient cells contain very much less mature miRNA compared to normal cells because the dicer protein functions as a key miRNA processing enzyme, RNAse III. So this cell line provides a good tool for studying the function of miRNAs. By introducing individual miRNA into these cells and avoiding multiple interactions of microRNA and mRNA it is possible to assess the mRNA levels in the cells and assess specific miRNA functions. Based on microarray profiling results, it is clear that the expression profiles of a number of genes in the cells were affected by some of the exogenous RNA sequences. The changes in levels of some of these affected genes' mRNA were verified by qPCR. The pathways enriched among those down-regulated genes were not affected by the insertion of two insect miRNAs, miR-263a-5p and bantam which suggests that the process of transfection itself was not the cause of the observed gene expression changes. This observation suggests that RNA sequences in plasma might have some biological effects on human cells. The most pronounced gene knockouts were the nuclear encoded mitochondrial genes including cytochrome C oxidase and ribosomal protein S25. So in evolutionary terms this may suggest some kind of hard wiring between the microbiome and the human, or mammalian, system.

Mikael Rydén: All the donors in your study were completely healthy I assume but given the inferred importance of the microbiota in diabetes and the development of diabetes, have you had any chance to look at diabetics or people for instance with say inflammatory bowel disease or things like that? Could some of the effects be related to the microbiota transmitting their effects through the miRNAs in plasma?

Paul Wilmes: Our initial pilot was in 10 healthy individuals, but we are looking specifically at type 2 diabetes - because as some of you know, there was this rather large study that was published in *Nature*[75] where they showed there was a mild dyspoiesis associated with type 2 diabetes and that that was an enrichment in opportunistic pathogens in the case of type 2

diabetes. We are actually able to detect all those species in blood, so we are trying to see if we can validate some of their findings.

***Philip James**:* Studies from the Netherlands[76] have shown that if people with insulin resistance and glucose intolerance have their gut microbiome's switched by colonic content exchanges then within six weeks you dramatically change their insulin sensitivity. So the big question is what are the mechanisms and are the miRNAs involved?

Plant miRNAs and their absorption

Howard Chang: I was hoping that somebody could educate me about using maybe experiments in c-elegans where I understood they first showed siRNAs in E-coli food induces gene silencing. If you also have these miRNA knockouts and then you copy them with wild type genes into some part of the worm, do they actually have systemic effects? Does it depend on the RNA application system and does it work for miRNAs at all?

Frank Slack: As far as I know, there are no data suggesting that miRNAs are transported from one cell to another cell in c-elegans. Experiments have been done where inexperienced technicians have injected siRNAs into the wrong place in c-elegans, and those do seem to get transported around. C-elegans, of course, are famously able to take up double-strand RNA from the environment, process it and transport it around the body, but there is no evidence that miRNAs get transported in c-elegans yet.

Chen-Yu Zhang. I think there was a paper published in *Nature Biotechnology*. They made a transgenic cotton with expressed siRNA and the insect eat this cotton and died[77]. And for the honeybee we already have found a specific phenotype on eating the specific plant miRNA where the worker bee could not develop their ovaries.

Jørgen Kjems: We have over the years worked hard on siRNA delivery in the gut, so we are bringing in particles with a labelled siRNA which can be fluorescent or radioactive and we see a very clear uptake when we feed these particles. If we have just naked siRNA, we have never been able to follow it anywhere in the body. But if we have it in particles - and we have tried a number of different particles - then some of them are quite efficiently taken up and we find full-length siRNAs in the spleen, kidney and liver. So there is clear evidence that you can bring in large molecules as an siRNA if you use the right particles. The evidence is clear.

Steve Chan: I am going to address copy number and possible plant miRNA uptake by animals which we recently published[78]. Our objective was quite simply to determine whether widespread delivery of diet-derived miRNA actually occurs in animal organisms after typical dietary intakes. So we looked at a few platforms: honeybee, mouse and human. We fed them a lot of different types of diet. We checked plant-derived miRNA in the honeybee after eating a season's worth of pollen and honey; in a mouse we utilised custom-made chow that was either enriched for vegetable and soy-derived miRNA or plant-derived miRNA or enriched in lard so that we had something like miR-21 in it. We also used ripened, fresh avocado as a diet to make sure that we weren't getting fooled by just looking at processed chow. Then we examined dietary records from healthy humans who eat a substantial amount of fruit during breakfast and just looked at their plasma levels.

Our first observation was that we could detect miRNA in the food sources - cantaloupe, orange, avocado, banana and apple - all carried substantial levels of plant-derived miRNA. We chose to study miR-156a, miR-159a and miR-169a as these are all conserved throughout the plant kingdom - they are pretty ubiquitous. We also looked at copy numbers in the fruit so that we knew how many copies per milligram of food we had. We found a little over a million or even greater amounts per gram i.e. 10^6 per milligram of food - up to 10^{12} per kg of food. This is very consistent with previously published analyses of fleshy fruit-derived products, and it is very consistent with what I think Chen-Yu Zhang actually published 2

years ago, when looking at rice. However, when we measured ham it did not have any of them, but it did have a substantial amount of animal-derived miRNA - we used miR-21 as an example. Again, I can't go through everything, but I will give you an example of the type of data that we obtained. After twenty-four hours' worth of eating an avocado diet, we were able to detect in stomach contents partially-derived avocado miRNA in amounts between 1 million and maybe 100 million/g. So we know that the animals ingested the miRNAs.

The problem is that we were unable to detect very high levels at all of any of these miRNAs in the plasma, liver, lung, kidney and stomach. MiR-159a or miR-169a, were undetectable in our hands but miR-156a was interesting because we did see levels, but they were exceedingly low. When we calculated it out, it was about a little over one copy number for every μl of plasma. Remember I mentioned endogenous miR-16 in our hands had about 140,000 copies per μl - so this is very different. Liver and stomach maybe had about a little over one copy for every 2 cells that we tested, so we used an approximation of 10 picograms of total RNA for how much is in one mammalian cell. So we calculated that there was about one copy in every two cells. For the lung or kidney it was even worse - maybe about 1 copy in every 10 cells for a discernible copy of 156a.

Eva van Rooij: Could you please specify how you determined the copy numbers?

Steve Chan: Sure: we utilised trizol-based extraction and taqman-based RTPCR. So we took synthetic plant miRNA for 156a, 159a and 169a and we made a standard curve so we knew how many copies we were putting in and we made serial dilutions and we were able to dilute it all the way down to the final copy number. So we were able to determine one copy number per reaction. Now it is a little bit difficult here to say that synthetic copies are necessarily exactly the same thing as the natural ones, but I think that we were able to develop the standard curve for miRNA amounts and we were able to detect substantial levels of these miRNA in the food. So we were confident that the technology we were using was valid. I have to say that since the time we published these data, there have been a number of investigators who have contacted me to say that they get the same type of data, but using different animal platforms and looking at different plant miRNA and in fact using different technologies. So they don't all use taqman-based assays; some of them have used next-generation sequencing; some of them use droplet digital PCR and the like, but all in vain. We actually did a number of experiments, all of them showing the same thing: occasionally we do see exogenous miRs in human plasma and tissues, and these may be derived from a dietary source - so we can't rule out that possibility. But I think that the real crux of the issue is that we don't feel that exogenous plant miRNA delivery is ubiquitous for all plant-derived miRNAs and that we, in our hands, find very little evidence for robust or widespread steady state expression for the miRNA that we tested in these recipient organisms. And the numbers present are actually quite low - less than one copy per cell in various organisms. Again, if you go back to 2007, there is a paper saying that at least 100 copies per cell is the prerequisite for canonical miRNA function[79.] So we are, I would say, roughly 10,000 to 100,000 off the mark in that case.

Kendal Herschi: Steve Chan's data are corroborated by the group at Monsanto who did the insect feedings. They also failed to see the plant miRNAs uptake so there is some consistency to that non-finding.

Eva van Rooij: We have found it is very difficult to detect plant miRNAs in a rodent.

Martijn Nolte: I just wanted to ask you, when you looked at the organs, did you perfuse the animals with PBS to get rid of the blood in the organs?

Steve Chan: We definitely did for the liver, lung and heart.

Ulf Smith: There are a lot of other things that are put into vesicles and circulate but we don't expect them to have any effects so is it just because it happens to be a miRNA that we assume that there should be an effect?

Kendal Herschi: I tried feeding my wife, children and other volunteers with a rice diet to look for miRNA-168 but I only see the miRNA at very low levels.

Patrick Stover: Steve, did you look in the mice where the miRNAs went - into the faeces or were they hydrolysed? Do you know their fate?

Steve Chan: We did look at faeces and we again saw some degree of miRNA and in plasma and tissues but at very low levels; the levels seemed not to correlate with the dietary amounts. We also looked at urine and that did not show much of a correlation either. I think that a lot of people are thinking maybe it is a deficiency in methodology but in our calculations we don't think that there are actually enough copies of a given miRNA per gram of food to get into the number of cells that are necessary. So we now know that there are about a million copies of miR-156a in a mg of food but 10^{13} human cells in the body. We also know that you need to get at least a 100 copies into each cell, so you do this very simple calculation and realise that you would need to eat around 1,700 kg of cantaloupe to get up to the right copy number in cells..

Irv Rosenberg: I just wanted to express my appreciation to Stephen Chan for returning us to the issue that dose is important - whether one is talking about copy number or the amount in breast milk, We need to be talking about the importance of these things functionally rather than pharmacologically. We do need to recognise that there is going to be an issue of dose or abundance in addition to things like complementarity. Obviously, in other forms of nutrition and other systems where there are changes in function associated with SNIPs it is possible to overcome some of these changes in function by increasing the dose of the nutrient or the pharmacological agent. I realise when you are dealing with knockout experiments, it is sometimes difficult to do dose responses, but I do hope that we can continue to refer to dose and exposure when we talk about these functions.

Esther Nolte-'t Hoen: As an immunologist everything in the immune system is about finding the right antigen. For example, if you inject systemically T-cell-derived vesicles, which are carrying the T-cell receptor, then specifically those dendritic cells are targeting and presenting the antigen that is recognised by those T-cell receptors. Then you get vesicle-specific effects on those dendritic cells carrying these antigens. So targeting is a very important aspect of the remote effects of the vesicles, and this targeting is very specific.

Chen-Yu Zhang: I don't believe that it is very hard to really obtain an accurate copy number. You may have a trebling in the miRNA of a very low concentration but his may be very physiologically important.

Irv Rosenberg: I would like to return to this meeting's question relating to the title "The Role of miRNA in Nutrition and Disease". Now that we have so clearly clarified the relationship between miRNAs and cancer, and miRNAs and chronic disease and regulation, are we able to take a look at the question of "what is in fact the relationship between miRNAs and dietary intake and nutrition?" Picking up on Bob Chapkin's point and perhaps Gerhard's point earlier, is there the opportunity here to look at miRNAs, or other forms of non-coding RNAs, that are in fact part of our diet, so that we can look at dosage in some of the same ways that we look at dosage of polyphenols, dosage of micronutrients? How these intakes or dosages have an impact on metabolism, regulation and so forth is then important. Are we ready to accept or reject the notion that not only is there exploding and fascinating information about miRNAs and their regulation of gene expression but miRNAs are part of our diet and do they in fact pass from breast milk to the child, and across the placenta? Where are we with that? Are we ready to include these among those aspects of diet and nutrition which are having a significant impact on disease prevention or risk?

Philip James*:* So, Ester, you described the selective packaging of miRNAs in breast milk. Have you actually measured those packaged miRNAs in the child after feeding the breast milk?

Ester Nolte-'t Hoen: No, we haven't yet. What has been demonstrated in about 5 papers is that the cells of the mother pass via milk into the circulation of the child,[80] and these cells will include all of their RNAs.

Chen-Yu Zhang: I think we also need to recognise that these miRNAs have a functional role. For example if I feed mice with a synthetic miRNA, we see the function in the foetus altered.

Philip James*:* You mean the miRNA is passing through the placenta?

Chen-Yu Zhang: Yes. This miRNA can be passed through the placenta. That is the first thing. And another point - some people cannot detect 168 after a lot of steamed rice but we can.

Frank Slack: I have never tried experiments of this type. In the biotech field, siRNAs and miRNAs are not thought to cross the human placenta, at least therapeutically. I am not sure if Eva wants to mention whether any of her miRNAs or antimiRs have been shown to cross the placenta? But it has actually been a limitation for mouse genetic studies in that you cannot RNAi a foetus, which is what some people would love to be able to do. You can do that in c-elegans - it works well and people thought that maybe it would also work in the mouse, but it doesn't, and I would suspect that that is also going to be the case in humans, although nobody has ever tried to RNAi a human foetus.

Chen-Yu Zhang: This miRNA absorbed by the pregnant mouse is basically in the microvesicle. This microvesicle can carry this miRNA and pass through the placenta

Philip James: Jørgen Kjems from Aarhus has already highlighted the fact that he has synthetic RNAs of a molecular length similar to normal biologically active miRNAs and he demonstrated that these could be absorbed[81], using similar oral techniques to those employed by Chen – Yu Zhang[82] in Nanjing, and has actually picked these miRNAs up in different tissues. So it seems to me it is quite clear that we have two groups here who are showing the capacity to absorb these relatively small molecules, apart from the whole breast milk story which is well-documented and understood in terms of the absorption of quite substantial cellular components. But what are you going to say to all our other colleagues who say they cannot find miRNA absorption? Kendall Herschi is quietly saying in the background that he has tried similar studies in terms of absorption of MiRNA from plant sources and cannot find them in the blood.

Chen-Yu Zhang: I will send my technician to his department! We have also tested miRNA from the Chinese herb. You can detect it in high levels in human blood after ingestion and you can detect it in the mouse lung and other tissues after giving it orally.

Kendal Herschi: I think this whole meeting is excited about this possibility, but to move it forward, we have got to replicate your findings and then move forward. So I want a road map, because we have looked for miRNA168, 156, and miRNA-161. So we haven't just looked at one miRNA. Steve Chan has also looked at three different miRNAs and we cannot show their absorption.

Philip James*:* But Jørgen pointed out to me last night that actually if you have got a very selective target and you get a functional change, that is meaningful.

Kendal Herschi: We are building bio-sensors for 168 and putting them in the liver. So we are a couple of months away from having a sensor for the miRNA in the liver. We have also got transgenic plants generated against blood clotting factors. We are also trying to repeat Chen – Yu's work with hepatitis. But it is difficult to remain enthusiastic. We keep doing these experiments unsuccessfully but it is just hard to go forward with this stuff coming out. So it is a good time to have this meeting.

Jose Ordovas: I am more on the Nanjing side. We are not using rice but olive oil and we are able to detect the miR-168 and we do it in a dose response manner - we load people with olive oil and then we get the dose response effect in blood.

Kendal Herschi But 168 is a miRNA that is mostly found in monocots so you can sequence the difference between the monocot and dicot variants. So if it is in the olive oil, you would think that you should be able to see the dicot or monocot version - I don't know what you have?

Jose Ordovas: we are currently sequencing to find out.

Chen-Yu Zhang: Actually, there are high levels of the miR-168 in the other plants as it is important for plant development. So the miR-168 is enriched at high levels in rice, but also with relative high levels in other plants.

Pier Paolo Pandolfi: We should really look at what is being given to whom and in which formulation. Olive oil is a different thing from plants and there could be that there are genetic differences in uptake. The detail is very important.

Kendal Herschi: But some of the papers that are reporting non-results in tests of miRNA absorption are saying that they tried mice and it didn't work in the mice. We have also tried in mice unsuccessfully. However, when we went to China, I looked at the chow formula that Chen – Yu uses and the chow formula in China was very different from the chow formula we feed in the United States.

Steve Chan: In relation to Kendal Hirshi's work we never looked in very much in depth computationally because I am not entirely sure whether the same exact rules apply when a plant miRNA and a mammalian MRNA are being assessed. We could certainly look at the seed sequence, and when we do that, sure a lot of genes pop up. However, when we did an initial network analysis it was unimpressive so we didn't take it much further.

I just wanted to clarify that whilst we actually published fairly negative data, I think everyone has to understand - and probably already do - that we were not exhaustive in our search. Our objective was really to determine if this was widespread, if it was frequent and robust, and we found that it was not. At least based on our data. That doesn't necessarily mean that it cannot happen, or it does not happen. We believe that it would have to be in a very context specific fashion, so that is in very specific packaging of unique miRNA in certain food-types. It would have to be perhaps specific gut cells that actually take up or concentrate the miRNAs. For example, the gut lymphoid tissue might be involved and perhaps that is a concentrating process that would allow for milk exozomes with immunomodulatory miRNA to get absorbed through this route. We can discuss all of these possibilities but the most important part about moving this field forward is to actually have peer-reviewed publications where we can really compare exactly what people are doing. Then we can really start to dissect out what the differences are and how we can integrate these data.

Frank Slack: A few years back I asked one of my graduate students to tell me if there are any miRNAs in what we eat that might target oncogenes that might be healthy for us. Perhaps things from tobacco that might target tumour-suppressor genes. She came back with the same comparisons that Steve Chan has made i.e. that many plant miRNAs, if you use the same predictive rules that apply to animal miRNAs, could target thousands of human genes. In fact, we couldn't make much sense out of it. Furthermore when she randomised the sequence of grape and rice miRNAs, we got back the same type of data implying putative action so basically that investigation stalled. I am thinking that maybe we ought to publish that now because maybe there was some interesting negative data there.

Then the last thing that I want to mention is a paper from Philip Askenase who spent years trying to find the tolerance factor involved in T-cell tolerance - and finally identified exosome-like nanovesicles in the supernatants of CD8(+) suppressor T cells that were not regulatory T cells and an RNA in the nanovesicles proved to be miR-150. A surface coat of antibody

light chains or possibly whole antibody from the nanovesicle membrane controlled the targeted delivery of selected inhibitory miR-150 to the CS effector T cells[83]. Basically, the miR-150 mice don't make this tolerance factor. If you isolate vesicles from miR-150 mice, and you artificially transfect them with miR-150 you can now restore the tolerance to those vesicles. So that is about as close as I have ever seen to anybody doing a clean genetic experiment to some extent complementing a mutant with the vesicle-loaded miRNA. He calls these things exosomal-like, because I don't think he really knows the exact composition of these vesicles.

Nutritional issues and miRNA in metabolic control

Jose Ordovas: I want to mention our work on how docosahexaenoic acid(DHA) modulates the enterocyte expression of microRNAs involved in lipid metabolism. Differentiated Caco-2 cells were treated with either vehicle, DHA, oleic, or palmitic acids for 24h and miRNAs were measured by quantitative RT-qPCR. Different miRNAs are either selectively enhanced or repressed by each fatty acid. We found a statistical increase in miR-30c by DHA. Gene ontology analysis of biological processes of the miR-30c predicted targets, using the GeneCodis3 software, associated with lipid metabolic processes and protein-protein interaction analysis using the String software suggested that the miR-30c selected predicted target genes related to lipid metabolism, particularly lipoprotein lipase (LPL)[84.]. We then found greater RNA expression in heart skeletal muscle and brain. LPL levels are partially lowered by miR-410 targeting of the LPL 3'UTR and inducing the rs13702 T allele of LPL. We performed a meta-analysis across ten cohorts of participants that showed a statistically significant association of the LPL variant rs13702 with triacylglycerols (TAG) and high-density lipoprotein cholesterol (HDL-C) with each copy of the minor allele associated with 0.060 mmol/l lower TAG and 0.041 mmol/l higher HDL-C. Our data showed that an LPL 3' UTR luciferase reporter carrying the rs13702 major T allele was reduced by 40% in response to a miR-410 mimic. Meta-analysis demonstrated a significant interaction between rs13702 and dietary polyunsaturated fatty acid (PUFA) with an inverse association between dietary PUFA intake and TAG concentration with a -0.007 mmol/l greater reduction. So this suggests that the rs13702 sequence induces the allele-specific regulation of LPL by miR-410 in humans. Dietary PUFA may modulate miR-410 in addition to LPL to further influence this reduction in LPL concentrations. LPL concentration is not affected by miR-410 in the presence of the rs13702 C allele because the miRNA doesn't bind, so there is then a relative increase in LPL. What we have seen is that similar changes from other polymorphisms that we are following have a very significant effect on cardiovascular disease risk.

Kenneth Chien: You are showing that lipoprotein metabolism can be influenced by miRNAs which clearly makes sense and then, if the miRNAs are getting in through the gut, they could be in the chylomicrons and influence regulation in the liver.

Jose Ordovas: We also see that the regulation of these miRNAs for example at the intestinal level is specific to different fatty acids and they are indeed packaged into the chylomicrons.

Kenneth Chien: Is this a specific subset of miRNAs that are always in the chylomicrons, and are they the ones that would have an effect on target genes that would somehow auto-regulate some function of the lipoprotein?

Jose Ordovas: It depends on the diet and the type of fat that you are feeding - you will then have a different set of miRNAs - so it gets to that level of specificity.

Susan Ozanne: A common function for these miRNAs seems to me to involve the regulation of growth. If true does it tell us something fundamental about the roles of miRNAs and gives us more of a rationale for why it may not be too surprising that they can be regulated by nutrition?

Gerhard Aihauld: If we put an animal on a high fat diet - 60% energy as fat - and at 22^{o}C instead of 28^{o}C, these are very, very stressful conditions in rodent nutritional circumstances.

With so many miRNAs involved, for instance, in adipogenesis or even in brown fat formation I am wondering if we can eventually have some kind of standardisation of nutritional conditions to see whether or not this is just a response to stress or a truly normal nutritional feature associated with development?

Mikael Rydén: We are working with white adipose tissue and we are still like kids in a candy store when it comes to miRNAs! I find it fascinating to see that in the cancer field you can select specific miRNAs that you can validate in so many different cohorts across the world. I wish we could do the same within white adipose tissue because that is simply impossible. It is very difficult to reiterate candidate miRNAs in different cohorts, maybe because as Gerhard said, we don't have the same conditions. I mean these stressful conditions of gaining and losing fat are very, very different, and there might be other differences as well. We are still at a very basic level and what we are really interested in knowing is what regulates the regulators? Why are miRNAs altered in expression? That is what I hope will be the next goal in the coming years, to understand better.

Gerhard Aihauld: The role of n-6 fatty acid in promoting inflammation is also important. For instance, in the US, if you analyse the breast milk fatty acid composition, omega 6 fatty acids have increased threefold over the last few decades.

Philip James*:* That is because breast milk not only comes from the current diet but two thirds of the fatty acids come from the 1% of the total fat mass recirculating as fatty acids liberated by lipolysis every day. So the breast milk fatty acids reflect a longer term pattern of fatty acid consumption.

Gerhard Aihauld: In our mice studies the pups will, of course, consume breast milk and then after weaning will be put on the same diet as the mother and father. And yet after just 4 generations, you get obesity with a high n-6 diet. Nothing else has changed on a quantitative basis, just a macronutrient qualitative change.

Philip James*:* There are new studies coming out which show, for example, that the greater amounts of - n-6 fatty acid than n-3 fatty acids measured in blood as a predictor of what was eaten by a woman in pregnancy actually predicts the adiposity of her child, at least up to the age of 6 years [85]. The carbohydrate/fat ratio diet in pregnancy was also shown to relate to epigenetic changes observed in umbilical cord at birth indicative of changes in the babe which in turn related to the children's adiposity at the age of 9 years[86].

Gerard Aihauld: We heard about vesicles in breast milk delivering a package of miRNAs - are these miRNAs all modified by virtue of all the n-6 packed into the adipose tissue?

Ole Hernell: I have to comment on the potential role of omega 6 in breast milk. You would think that the most relevant omega 6 fatty acid in terms of inflammation would be arachidonic acid and that is actually more restricted in breast milk than other fatty acids, so even if the mother has a very high intake, you don't change the concentration of arachidonic acid that much, which is in contrast, for instance with docosahexaenoic acid [DHA] which you can actually change quite considerably. If you look at young children and go back and look at their early diet and see what in the diet is the most striking risk factor for developing obesity later, it is not fat - it is not the fatty acid composition, it is the protein intake.

Howard Chang: Have you looked at whether you were able to transmit the effect of obesity post diet just with the sperm of the father exposed to that diet and a basically normally fed mother and then with the offspring on a normal diet? Some of these effects have been observed before and that would very clearly show then it is an epigenetic effect that would be trans- generationally inherited. The relevance to this particular meeting is that there are two proposed kinds of mechanisms that carry that kind of trans-generational information. One is obviously DNA methylation in sperm; the second is usually a class of small RNAs that are in sperm and they are believed to cause changes called paramutations. So these are non-Mendelian effects that can be transmitted through, it is believed, small RNA in sperm.

Gerhard Aihauld: We tried to get rid of any genetic influence of any sexual differences, so we crossed males and females at random, just to prevent any genetic influences. So the study was purely designed to look at dietary influences and tried to exclude as much as possible any genetic influence from either parent but, of course, that does not deal with epigenetic or other effects you cite. We have not looked at miRNA in these studies,

Chen-Yu Zhang: Actually, 40 years ago some analogous experiments were done. In a very famous trial involved the carp fish with one tail and the golden fish with two tails. Two Chinese scientists basically extracted RNA from carp eggs and then injected this RNA into the golden fish eggs. One in three of the first generation had one tail and interestingly the f2 and f3 also have one tail. At that time people said this was pseudo-science, not real science. But we think we should re-check this kind of phenomenon and see whether this is miRNA mediated or not.

Pregnancy and epigenetics.

Gerhard Aihauld: In dietary terms we had published, in collaboration with Pascal Barbary, an analysis showing that a moderate fat diet of 35% with an n- 6/n-3 ratio of about 27(the US average ratio is between 17 and 40) led, after 4 generations, to all the animals becoming obese and this relates to the amplification of several genes involved in adipogenesis. So we are seeing profound adaptation over a few generations which I think must involve miRNAs but we have not yet studied these changes in detail [87],[88].

Philip James*:* But Gerhard, what most of us need to realise is that we have changed the national dietary n-6/n-3 ratios were progressively increased in many Western societies after the Second World War as part of the cardiovascular prevention measures whereas traditionally the n-3s in vegetables and fish have been a relatively high proportion of the total polyunsaturated fat in the food chain. But Ancel Keys and others in the '50s / '60s discovered they could drop LDL cholesterol levels by pumping into the diet n-6 fatty acids. N-3 fats were not favoured because with the development of the food industry using higher n-3 rich products reduced the time before products became rancid. So there was also a progressive reduction in n-3 levels as n-6 levels rose. We know that the n-6 fatty acids produce during their metabolism pro-inflammatory prostanoids rather than the anti-inflammatory prostanoids derived from n-3 fatty acids. Now Gerhard is adding an obesity problem to the concerns about the high n-6/n-3 ratios in the diet.

Chen-Yu Zhang: We have found that pregnant mothers' miRNA can pass through the placenta and then influence the foetus's physiology and dietary miRNA in mice can pass through the placenta.

Sue Ozanne: I just wanted to highlight as well, in terms of these trans-generational effects - not just the mother's diet during and before pregnancy, but actually the grandmother's diet because you have got to remember that those mothers' germ cells themselves were being established in the grandmother.

Philip James*:* Is there evidence for that proposition in humans?

Sue Ozanne: In humans there is emerging evidence: it is not as strong as it is in the animal models, but there is emerging evidence which is becoming stronger. The other issue is that miRNA biomarkers can be induced to change by a diet in one generation but subsequent dietary changes may be to little effect So if it is just the exposure during the grandmother pregnancy, trying to link outcomes in the grandchildren to what her daughter eat before and in pregnancy may not be relevant. So it just makes it much more complicated.

Philip James*:* Yes but not only have you got this inter-generational effect, but you have also got the dramatic effects of the actual pregnancy itself. Data from India, for example, show the astonishing rates of developing diabetes if that babe in fetal life was put through tough times. But goodness knows what the grandmother was put through. It is fascinating

because the susceptibility to all these metabolic diseases like diabetes and hypertension is 4-5 times greater in relation to any standard body weight status when one compares Mexican with US whites or Indians with Australasians. I have been able to superimpose the diabetes/BMI relationships of Mexican data on the Indian and Asian data and it is absolutely consistent. So are we dealing with some intergenerational effect and that most of the world was malnourished 50 years ago? We are now imposing extraordinary environmental conditions on mankind which has been semi-starved for millennia.

Sue Ozanne: Agreed but there are now at least two studies looking at children born to women before as well as after their mothers had bariatric surgery. This showed the beneficial effects in offspring born after surgery compared to those born before, which means that we do have the potential to have relatively big impacts during the current pregnancy. A paper in press[89] comparing offspring of the mothers before bariatric surgery with their children after surgery revealed links between gene methylation and expression levels correlating with plasma markers of insulin resistance (fasting insulin and homeostatic model of insulin resistance). A total of 5,698 genes were differentially methylated between siblings born before their mothers had bariatric surgery [BMS] and after surgery [AMS]. These siblings, exhibiting a preponderance of glucoregulatory, inflammatory, and vascular disease gene changes, showed a statistically significant correlation between gene methylation levels and gene expression and plasma markers of insulin resistance consistent with metabolic improvements in the AMS offspring. This reflected changes in the genes involved in diabetes-related cardio-metabolic pathways.

Philip James*:* the Institutes of Medicine in the United States has been highlighting the importance of really getting women's weight down because even within the normal range, weight range of women going into pregnancy predicts certain outcomes for the babe. So we are dealing with extraordinarily sensitive phenomena that we are far from clear about? Are we talking about regulatory systems involving miRNA?

Curt Harris: I think we have to be very careful when we say epigenetic. Is it DNA methylation or Is it miRNA? There are lots of epigenetic changes and markers in chromatin and studies of ageing in worms show that they have epigenetic changes. So I think we have to be a little more precise.

The role of miRNA in Therapeutics

Kenneth Chien: One of the big interests in miRNAs is as therapeutic agents. There are companies that have invested millions of dollars in these developments and what I hear so far is that there is a class of miRNAs that is quite specific to a particular cell, so they could be logical targets because of their specificity. But then the challenge is whether they will have off-target effects directly or indirectly? There is a lot of evidence, it seems to me, that these effects things are combinatorial, that they affect multiple genes in multiple cells with multiple targets serving as a fine-tuning mechanism rather than acting as an off or on lever. Now if this is true, then the effect you are going to see is rather incremental, and won't be pivotal enough to see an effect therapeutically. Then you have the same history with RNAi, where there was a lot of hope followed by hype with huge increases in the stock price of particular companies before they crashed. So my question is: are miRNAs ever going to be strong therapeutic candidates with wide application? If so, what are the best diseases right now, given the properties of miRNAs? And what do we need to do to get them to the next level - that is what I would like to know

Robert Chapkin: We are focusing a lot on what modulates miRNA's impact on biology, but I believe there was a seminal paper from the Whitehead Institute that showed, at least in malignant transformation, that there is a universal shortening of untranslated regions[90] and therefore the sentiment was "don't just worry about the miRNAs changing, it is the messages

that are also changing under these conditions". Therefore the response of the entire system is being perturbed as a result of a whole new steady state.

So when we consider obesity and chronic heart failure: are these also steady states where something remarkable is happening in the untranslated regions and not just coming from the miRNA dimension?

Eva van Rooij: The miRNA therapeutic field is advancing rapidly and helped by all the lessons that we learned from the SiRNA field. There are already very good chemistries now being tested and used. I agree that the more specifically expressed miRNAs are going to be better targets. The target specificity analyses show a potentially therapeutic miRNA targeting many different miRNAs. The most specific targets are probably going to be the most relevant genes that are going to be assessed by regulatory authorities. For more chronic therapy we are going to have to be a little bit more careful.

Frank Slack: When considering therapies I might seem slightly conflicted as a consultant to two companies in this area and we also hold patents on using miRNAs to control oncogenes and miRNA to control insulin signalling. So the question has come up as to whether these approaches might make it into the clinic, are effective or whether it is better to target only very specific sites. The first question of whether we are going to make it into the clinic has already been answered because they are already in use. Their effectiveness has also already been partially answered. For example, the miravirsen, which is an antimiR-122 antisense molecule that is effective against hepatitis replication[91,92] has the added advantage that it also affects cholesterol levels when tested in monkeys[93]. I am not sure whether the humans also had a reduction in cholesterol - I think they did.

There are two sides to the therapeutic coin: there is the targeting of the miRNAs that might be overactive in a disease and in that case you want to use antisense siRNA type technologies to knock down those particular overactive molecules. Then the other side of the coin is restoring miRNA function to cells that have lost those particular miRNAs. A couple of very non-specific examples: we have heard from Curt Harris about miR-21. It is actually expressed in many cells, and yet the mouse knockout mutant almost has no function, as far as we can tell right now until, of course, you stress the animal. So that has led a number of therapeutic companies to think that miR-21 will be an effective target because in certain diseases, and particularly in cancer, miR-21 is massively overexpressed. So if you can deliver this antisense miR-21 molecule to every cell most cells are not going to care but the cancer cells are likely to respond. Another example is miRNA let-7 which is also expressed in almost every differentiated cell but cancer cells have lost their let-7. So here is another case where you could deliver the let-7 mimic to every cell and they are not going to respond given their routine exposure to let-7 whereas the cancer cells will now be exposed to the miRNA. So these are the two examples that I know of that are closest to the clinic for cancer therapeutics.

Philip James: how do you deliver the miRNA?

Frank Slack. If you are trying to knockout a miRNA, you have to use a different type of delivery than if you are trying to restore a miRNA where you have to provide the cell with an RNA structured molecule that the argonaute can actually recognise and incorporate and then use as if it was transcribed within that particular cell. So you need to deliver an RNA molecule. In general, the molecules are delivered through some sort of nanoparticle and at the moment the ones that are in clinical trials are being delivered through lipid nanoparticles, and they do tend to go everywhere - at least the best ones go everywhere. There is little clinical activity with targeted nanoparticles right now. If you want to knockout a miRNA, then you can use a modified RNA molecule that is stable without a nanoparticle; in fact miravirsen and anti-mir-21 molecules are actually lna type molecules which are very stable: they get injected into the patients without any delivery vehicle and they are very short; they survive in the blood stream because they don't have any chemical bonds that the body knows how to degrade. And they can get into cells to just bind to the natural RNA molecule and stop it

from functioning. It doesn't have to fool the cell into thinking that it was something that was made within the cell - it just has to act like a drug to bind to the natural RNA.

Eva van Rooij; I am also involved in a company but I agree with Frank that for the targeting of miRNA, you should preferably have a more specifically expressed miRNA. or you are better off having a more specifically expressed miRNA.

Curtis Harris: These are excellent points the companies also highlight. However we need to recognise the importance of considering the dimensions of side effects. If one is dealing with a side effect with a cardiovascular impact or a chronic disease/metabolic risk profile then the acceptable tolerance is in practice near zero for side effects whereas with cancer there may be nothing else one can do so miRNA therapy with some side effects becomes more acceptable. Also my understanding of these lipid particles for delivering miRNA is that they tend to go to the liver and so for liver diseases e.g. hepatitis it kind of makes sense to consider the potential benefits of a miRNA. But if you are targeting pancreatic β-islet cells that is much more difficult, particularly when giving repeated doses of a lipid carrier when some unwanted signalling seems to occur. So I am not saying the approach will not work and the most promising arena I think is where one has a missing miRNA that one is trying to restore. Whereas if you are trying to engineer a loss of function of the miRNA this involves the injection of a compound which has to be sustained at a tissue level with high enough concentrations to be effective. So the half-life becomes an important consideration.

The real challenge is therefore whether one has a miRNA therapy that can be shown to be superior to conventional therapy. Where is the niche for it that is compelling? There are many challenges with its use for major diseases.

Roderick Dashwood: I agree that in cancer therapy reintroducing let-7 or knocking down p-21, really aims to inhibit cell proliferation or trigger apoptosis. But if you have another disorder like neurological disorders, that already have excessive apoptosis, then this would be a deleterious outcome. So in cancer therapy with histone deacetylase (HDAC) inhibitors we are triggering apoptosis through gene de-repression so one then needs to be careful about any propensity to neurological disease.

Chen-Yu Zhang: We should not emphasise too much the specificity of a miRNA's function. Then for the delivery system people always use artificially produced nanoparticles but we should consider using the normal biological micro vesicles which may not be so target specific. Nevertheless by inserting a miRNA or siRNA into a tissue then the organ will package the miRNA into a micro-vesicle which can deliver miRNA or siRNA sufficiently into a tissue. For example we have found a major link between tumour secreted miRNA-214 and the suppression of the immune system via a Tregs mechanism which induces IL–10 secretion and the expansion of tumour growth. However, by the use of micro-vesicle delivered anti-miRNA – 214 antisense oligonucleotide this blocked the Treg expansion and tumour growth[94]. So experimentally one rescues the T-cells from immune suppression by the tumour and this involves a new pathway between tumours and the immune system. Another system we are working on involves the insertion of a siRNA into the T-cells which can then shut down HIV[95]. This type of drug, has already finished the pre-clinical trial phase in China. I also work with a company.

Jørgen Kjems: I am also the founder of a company that is running nano-clearance and using miRNA technology. I think one should consider that all the data that we see now in the trials are the first generation developments of these technologies. By the 2nd and 3rd generation in coming years we will have a much more robust approach. If you look at the siRNA delivery, then there have been problems with targeting seed sequences often with degenerate pathways. So that means that you can often have a much better effect if you target miRNAs directly. Therefore there is now a shift of the field from siRNA to miRNA targets and this actually is very healthy. I think there will be a much better opportunity there.

Mikael Rydén: The problem we have in diabetes, of course, when it comes to using drugs, is that the first line drug of choice we have is metformin, which is a derivative of phenformin which was registered at the end of the 1950s, and this is the only drug where we even have a hint of its effects in terms of hard end points. All the subsequent drugs have an uncertain effect on the cardiovascular end- points. So it takes a very long time to see the effects of drugs acting on diabetes if we are looking at long-term benefit. So the same challenge will apply to the use of miRNAs in this field because again it may take so many years to see any effects on cardiovascular endpoints and I foresee large difficulties in launching them in the clinic. We still have the problem with the incretin therapy where there are safety issues, and nobody knows whether they really affect heart attack and stroke rates.

Ester Nolte-'t Hoen: Engineered vesicles, of course, are an option for creating a delivery particle for miRNAs or siRNAs. Our knowledge about these vesicles is still limited and it is rather difficult to create something that is clinically safe because of the large heterogeneity of vesicles. So we might have adverse effects that we cannot really control very well. So there is a whole research field now underway to really investigate how the extracellular vesicles deliver the RNAs to the cell and to really look for the key molecules that are involved, and then incorporate these molecules in the artificial vesicles which may in the end give a far more safe particle that then is clinically more applicable. It involves having specificity in targeting and in the endosomal escape of the siRNA into the cytoplasm where they must associate with the RNA-induced silencing complex (RISC) to direct the cleavage of mRNAs bearing complementary binding sites. In particular, the trafficking of siRNAs from endosomes into the cytoplasm represents a major rate-limiting step for many delivery approaches[96]. So that is why many groups are focussing on how to optimize these delivery approaches and increase their efficacy.

Bo Angelin: I think the idea of using an organ which is reasonably accessible, like the liver, as a factory for producing things that should then reach their targets is probably much easier compared with actually trying to target a site or function directly. And the other thing relates to what Mikael Rydén was highlighting - the difficulty of finding very specific effects of drugs. The drugs that actually work best - like metformin that we have actually used for 25 years or more – are in use despite the fact that we still don't know how it works so well. I think the secret is that they work via multiple mechanisms and that is why the use of miRNA might be more interesting – the general effects of a miRNA maybe more interesting than very specific targeted molecules because they may actually work in a more balanced way. So on the one hand it will be very important to do these early studies and see if our thinking is right with some miRNAs having multiple functions - I think we will see a lot of backlash after the use of very specific, beautifully engineered and well-thought out therapies. They are so specific that they may also give us very clear side-effects: nuclear receptors targeting is the perfect example of this potential problem.

Leif Groop: Of course we have had metformin for years, but we should remember that no treatment thus far has actually been able to change the course of the disease, so there is no treatment for type 2 diabetes which really deals with it as the course of the disease is still a chronic progressive deterioration. Another type of drug we need is that which simulates the marked increase – often trebling – in insulin sensitivity that occurs in pregnancy Then, of course, we have the problem of the government regulatory procedures for ensuring safety which means that the areas of diabetes and metabolism will be quite a tough area for miRNAs at least on the basis of our current knowledge.

Kenneth Chien: I believe that there will be miRNA drug therapies but with antibody therapies it took us 20 years to really develop a viable therapy - it wasn't until the humanisation of the antibodies that we were really able to make progress. I am involved in an investment fund that invests in companies so my caution is matched by a desire to see this come to a successful conclusion. One of the things that is very difficult to do is to improve a drug if you don't know what its exact target is so you may need to engineer several generations of product before getting there. With antibody therapy: we started out

with Herceptin and now Genentech (whom I advise) has been able to engineer a payload onto the antibody with remarkable effects. But this involves the antibody taking it to exactly where one needs to go and it then takes out the cancer. So I just think if you don't have that level of specificity of targeting then it is difficult to see effects without a lot of side effects. Humanised antibodies can be given chronically and, we know, relatively safely. So you are going to see these antibody approaches to ensure specific targeting come into even fields like cardiology where they already have encouraging results in altering blood cholesterol levels. So I think the key here is finding out what properties of miRNAs exist now and with some chemical modifications to match a unique clinical niche one might then expect the specificity and safety that we are all looking for. I think the hepatitis and cancer fields are very interesting; but I think the idea of these therapies doing a lot of different things is worrisome.

Use in Cancer Studies

Curt Harris: I work for the US government so I am under no circumstances allowed to work for a company but I think the interfering RNA story is not a valuable use of miRNAs. The second point is that, so far, the effects of anti-miRs look very modest in the clinic. As a physician treating cancer patients we put them through terrible ordeals in the hope of stemming the extension of the cancer. So this colours my view when assessing the use of this technology in cancer patients. In practice in cancer we do not assess the value of competing therapies because we are always trying a combination of therapies for greater effectiveness. Combination therapy has been around for a long time and we use this approach with chemo-resistant cells. Unfortunately the drug companies don't like to cooperate with each other and they don't like the idea of having multiple drugs being used. The combination may also be quite toxic when we use multiple drugs to inhibit those rare cells that are going to escape the original therapy. So something like anti-miRs I think will play an important role, along with the other range of remedies - antibodies, chemotherapy, radiation therapy etc. So I am quite optimistic about these general approaches.

Pier Paolo Pandolfi: I would like to make 3 points. Of course a miRNA has multiple targets. I work on phosphoinositide 3-kinase (pi3k) which is a kinase which has more than 200 targets. Many molecules have multiple targets and if we take p53 – as a very important human suppressor - we might think if we were able to deliver p53, it would cure cancer. So what I am saying is that this idea of pleiotropic effect is moot - it applies to all biology. The second issue is what is a drug target? Is it a target that drives the disease? Do we have an example that a specific miRNA drives a cancer? We are about to publish 2 Cell papers, back to back, to show that one miRNA can drive leukaemia[97] and the other breast cancer[98] to the metastasising stage; a single 22 base pair long structure is markedly over-expressed in cancer, so you want to take it out because it drives cancer.

The last point I would like to make is dependency: it is crucial to assess dependency. If the miRNA is a bystander, if you take it out, the disease will not budge whereas Frank (Slack) has shown beautifully that in certain instances, not only can you drive cancer with miRNA, but if you shut it down; the cancer is stopped. So, again, specificity and potency comes from the efficacy of that miRNA in that disease. The very last point in terms of drug development: once one identifies an miRNA that is crucial to the cancer then you have a 7 base pair long molecule you can immediately use as a target with rapid drug development This potential speed for drug development is unprecedented:

Frank Slack: Ken asked whether are some features of miRNAs that make them particularly compelling as drugs and drug targets? I would say that the one issue that hasn't been talked about is the fact that because miRNAs have multiple targets, it is like a one-drug cocktail. You are giving a single drug that is affecting many different things and basically

that allows you, I think, to circumvent the issue of resistance, so even the best Genentech drugs basically fail after just a few years in patients because the patient has essentially become resistant because this drug is targeting one single protein. So the gene for the protein just has to mutate and the drug is no longer effective. So I think having this complex multifaceted mechanism, is going to work to our advantage in certain diseases like cancer. Now whether it is going to work necessarily for diabetes as a chronic delivery system over the rest of the person's life, I agree, leads to some scepticism. But for cancer where the patients are essentially willing to take something that might be harmful long term, but short term gives them a few extra years of life, I think means that there is a good case to be made for miRNAs.

Curt Harris: There is another point and that is that most drug companies are not interested in a drug that doesn't make them billions of dollars and that costs the patient thousands of dollars a year. I am talking about the US. One of the questions with drugs that use antimiRs is whether they are really going to be nearly as profitable as humanised antibodies.

Philip James*:* Curtis I was very impressed with the scheme that you put up where you had certain predictors of cancer morbidity, recurrence and mortality, based on numerous cohort studies where you had identified specific miRNAs even in early cancer which help you to predict the likelihood of metastases development and mortality. Is that right?

Curt Harris: I gave one example, but there are other examples that are emerging. So the idea that you can cause a cancer, in this case, by over-expression of a particular miRNA, and that if you turn it off, the cancer goes away - I think that is a pretty compelling argument for that being a driver that is an important therapeutic target. I also agree that its use for drug development should be rapid. What you then need is to deal with the question of toxicity over longer time periods. So the questions for miRNA analyses in cancer are: is it a marker? Is it mechanistically a biomarker? Is it a therapeutic target? Will it have the suitable lack or minimum of side effects for the particular disease that you are treating, and is it cost-effective?

Philip James*:* The miRNA may be a beautiful marker of a specific cancer and a good prognostic index but is it therefore a therapeutic target? Now if you have got multiple effects then albeit that you would use it for cancer treatment, you might have a whole range of other metabolic effects?

Curt Harris: I am actually not so interested in biomarkers as those that have a mechanistic import in playing a role in the disease that you are studying. What is so attractive about miRNAs is that this next decade will show us whether we really have completely new opportunities. The therapeutic target is quite clear, and the fact that it has multiple pathways following from that target, that doesn't worry me at all!

Philip James*:* So the field is now at the point where multiple screening for miRNAs in the blood or tissues in different types of cancer patients is in progress?

Curt Harris: We and others are looking at cohorts and studies where we assess whether when you remove the tumour does the biomarker go away? Does it reappear at the time of recurrence? Does it predict who has micro-metastases and who doesn't and who needs different kinds of therapy? So there is a lot of work to be done in the near future, but the pathway - the road map - to doing those kinds of studies is quite clear. It is going to take some time, money and effort but it is no longer much of a mystery as to what to do now and over the next decade.

Chen-Yu Zhang: We find 3 different types of miRNAs are altered in disease. The first two you can find from the biopsy tissues. Why is it related to the on-going disease? For cancers there seem to be some cancer-specific miRNAs such as miR-21 and miR-214. Then there are tissue-specific miRNA such as for lung cancer. In lung cancer, a lung-specific miRNA is evident – also in kidney cancer. The third type of altered miRNA is the immune system's

response to the disease and released miRNAs. This type of miRNA, such as miRNA 223, miRNA 155, reflects the immune system's response to the disease.

Curt Harris: I don't think that is true! MiRNA-155 is produced in many types of epithelial cancers, and is probably a therapeutic target. I am not so interested in biomarkers of any type that do not have a mechanistic import. There is an immune response and you have lots of things happen: there are cytokines that are produced, and chemokines and so forth, and also changes in miRNAs. I consider miR-155 as actually causing leukaemia and lymphoma. In addition, it looks like it is very important in colon cancer, for example. And our very first paper on lung cancer showed that it was highly expressed in lung cancer, so it is a matter of specificity as a disease-related probe, as opposed to a reaction to disease. I am interested in what causes the disease and how to treat it.

Chen-Yu Zhang: My point is that there is an advantage if one can assess a circulating miRNA as a biomarker for the diagnosis, prognosis and even for the prediction of disease severity. So that is 3 different types of miRNA in each disease. For example, if you want to see whether it is a cause of the disease, of the altered miRNAs I would say 214 is mainly secreted by the cancer cells, not only one type of cancer cell. I screened 8 types of cancers: breast cancer, oesophageal cancer, lung cancer, liver cancer, pancreatic cancer, colon-rectal cancer and ovarian cancer[99]. I think we do need to think about the immune system because this affects cancer development and outcome. We[100] can deliver antagomir of 214 to the T-cells and we have already finished a pre-clinical trial to anti-miRNA-214.

Kenneth Chien: You want the marker to be biologically important, driving the disease, so that it is more than just a passive marker, it is actually involved in disease progression, and hopefully even a target. So, for example the amplification of human epidermal growth factor receptor 2 (Her-2) in breast cancer and other cancers can be used as a marker but also for therapeutic purposes with the use of trastuzumab (Herceptin) and analogous compounds. Dr. Pandolfi have you found an amplification of a miRNA in a specific tumour and is it gene amplification?

Pier Paolo Pandolfi: Yes, there are examples of genetic amplification of the locus. Mir-21 is an example - but there are examples also of a miRNA marker where the expression of the miRNAs are clearly driving the disease and again. There are also examples of dependency because if you take them out in mouse models then the cancer collapses. Of course, the caveat is that this is in a mouse study and that the cancer was initiated by that miRNA.

Kenneth Chien: OK, but is there any spontaneous naturally-occurring human tumour where miRNA is genetically amplified and then if you take that out *in vitro*, or in transplant, in ex-plant, you can then kill the tumour? I know in pancreatic cancers it can be difficult for even conventional chemotherapy to penetrate the tissue. Is there any evidence in these types of tumours that the miRNAs or the antagomirs are going to have difficulty getting to the tumour *in vivo*?

Pier Paolo Pandolfi: For pancreatic cancer I don't know. I know that kidney tumours maybe a desirable target because the locked nucleic acid [LNA] anatgomirs tend to accumulate in the kidney. So there are tissues that are more accessible than others.

Philip James: Frank says no!

Curt Harris: With pancreatic cancers, one of the problems is the hydrostatic pressure due to the fibrous nature of the tumours and at the American Society of Clinical Oncology (ASCO) meeting about two weeks ago a therapy that reduces the hydrostatic pressure in addition to traditional therapy looks like it is better because the drug penetration is the key. So there are ways of getting around this problem of pancreatic cancer, at least partially.

Kenneth Chien: Delivery is still going to be an issue I guess for any drug.

Cancer Prevention

Robert Chapkin: Since miRNAs work by multiple mechanisms this can be seen as making their use impossible but we have to remember that in nutritional terms nutrition effects are pleiotropic and complex and have a broad impact e.g. in epigenetics, energy metabolism, metabolic and hormonal control etc. Some experts with their focus on single mechanisms cannot cope with this complexity. We already know that disordered nutrition is, as most agree, of overwhelming importance in explaining a range of global killer diseases. So I am sceptical when it comes to all these drugs. A very powerful component of nutrition is its very important role in the prevention of disease, and most clinicians agree that we are going to make a minimal impact with drugs: the majority of people are going to have health benefits from prevention and this is where nutrition plays a huge role. So I would like to see people discuss miRNAs from a prevention standpoint. Are there ways to manipulate them because long-term chronic administration of any therapy is going to be really scrutinised and it will take years before we know whether there are indirect effects of a drug or miRNA that are undesirable, and many law suits probably as a result. So prevention is the way to go.

Philip James*:* If you look at the epidemiology of cancer, we often neglect the astonishing differences in rates between say the UK or US and the far lower rates of some cancers e.g. breast and colon cancers in such countries as China or Japan. Then migrant studies show a rather rapid emergence of colon cancer and the slower increase of post-menopausal breast cancer. So the question is how is that coming about and what are the preventive processes? When we use cohort studies as in the analyses of the World Cancer Research Fund[101] everybody gets excited by a 10-20% difference in the cancer rates in different groups whereas there was a 10-fold difference when we looked at rates across different societies. Are these differences diet related and what are the mechanisms involving miRNA that account for these differences?

Roderick Dashwood: There are quite a large number of dietary phytochemicals in different classes: isothiocyanates, indoles and so on that are known to have anti-carcinogenic activity and which work through epigenetic mechanisms affecting DNA methylation and histone marks. Some of those genes that are heavily methylated in cancers are genes for miRNAs. So I think we are going to see this interface of three elements of the epigenetic trinity: the chromatin modifiers, DNA methylation and the non-coding RNAs, including link RNAs and how they all relate to dietary factors.

Curt Harris: How much of disease-related obesity - including cardiovascular disease, neurological disease and cancer - is related just to inflammation? And is that the key or growth factor induction implicated here? Regardless of what causes inflammation, whether it is gastric reflux in the oesophagus or obesity or whatever - cigarette smoke in addition to carcinogens induced inflammation - this is what we need to look at now in terms of carcinogenesis. The implications are we should all try to lose weight and have a better diet.

Kendal Herschi: Surely we should be looking for biomarkers in all these sera collections of patients after bariatric surgery where weight reduction is not achieved really by simply lower caloric consumption.

Leif Groop: There are quite a lot of studies on biomarkers for predicting the outcome of bariatric surgery. I don't think that there is any unifying marker - the best are the branch chain amino acids where in all studies their changes seem to relate to changes in insulin sensitivity but whether this predicts weight loss I do not know.

Mikael Rydén: We are actually involved in a large Innovative Medicines Initiative (IMI) consortium[102] of public – private partnerships where the actual goal is to try to identify new biomarkers that would predict disease outcomes. Now what has come from the SOS Study - which is the largest bariatric study in the world - is that the best biomarker for outcome is insulin – a simple measure but hardly specific. However, there are 38 susceptibility genes for type 2 diabetes that we can measure but the assessment is very expensive and in

practice it is much cheaper and faster to ask your patient if their parents had diabetes and to look at the waist circumference of the patient. Those two factors predict diabetes outcome much better than the 38 susceptibility genes.

Steve Chan: A couple of comments on circulating miRNA versus tissue-based miRNA as biomarkers. One of the problems with circulating miRNA as biomarkers is that most of the time we are looking at plasma as a sort of dumping ground for most systems in the body. And a lot of the highly expressed miRNA that we found are expressed everywhere in the body, so if we use miR-21 as an example we have studies from healthy, collegiate athletes at Harvard who exercise for 30 minutes and afterwards they had elevated miR-21 levels in their blood stream. There are also a lot of papers talking about the inherent variability of circulating miRNA studies. So the problem I think in terms of using miR-21 as a circulating biomarker for cancer would obviously be: what is your control population? Have they exercised recently, what has their diet been? Perhaps there are diurnal variations in this marker. But I like the idea of using a biomarker in a tissue because then, as we know, it is actually driving the disease. So these values carry a lot of weight..

Chen-Yu Zhang: We don't use the one circulating miRNA as a marker for a disease; we use a combination of miRNAs. I agree miR-21 is not a good one - we never use miR-21 as one marker for predicting or diagnosing a cancer. Normally we use at least 5 miRNAs in combination, as a biomarker for the diagnosis of a specific cancer. The second point is that there is no standardising process for different labs to check their assays. This is the problem also in the companies that develop diagnostic kits based on the circulating miRNA

Roderick Dashwood: We have just published a review article on dietary phytochemicals, antioxidants, micronutrients that have been reported to impact miRNAs in various ways[103]. Many of the papers are very descriptive, and unable to distinguish cause and effect when considering the process of carcinogenesis. Is there a sequence of miRNAs becoming disregulated, much like the Vogelstein model for genetic development of colon cancer: you have β-catenin and K-Ras, and p53, although 20 years on we know that that is over-simplistic as only 7% of of cancers follow that precise model. We know, for example, that the loss of let-7 is occurring quite early on - so when we compare tumour with adjacent normal tissue, we are seeing a difference there, but even right after the dosing with the carcinogen, we are already starting to see loss of let-7 family members. So I think to me one of the most important players in this field is Alberto Izzotti[104,105] in the University of Genoa who is showing that with mice, he can expose them to genotoxins from cigarette smoke and he can measure carcinogen DNA well before they get lung cancers. This is associated with the dramatic loss of many, many miRNAs in the overall profile. If he exposes the mice to specific phytochemicals like n-acetylcysteine or phenylethyl isocyanate then he can reverse that process. Now he is asking mechanistically where the specific players in the miRNA processing machinery are and showing that these phytochemicals are preventing the processing and up-regulation of miRNA 21. Some of the compounds we have looked at like the isothiocyanates - sulforaphane is an example – stimulate research in the role of the NFR2 signalling pathway and gene activation. In our studies on phytochemicals with RNA-sequencing we are finding repeatedly that two thirds of the genes are down-regulated and those are usually non-coding RNAs.

Curt Harris: We showed that mothers and fathers who smoke during their pregnancy have children who, even though they were non-smokers as adults and had only at most passive smoking exposure, they were still at an increased risk of developing lung cancer. About 10% of those coming into our clinic today have never smoked. The children at highest risk have a genetic predisposition and that involves a hyperinnate immune system due to a mannose-binding lectin[106]. About 15% of Caucasians have these genetic polymorphisms that relate to mannose-binding lectin related immunity. So I think that if kids are exposed even in utero then there are epigenetic changes that occur.

Philip James*:* Are you are specifying this is not a post-natal passive smoking exposure?

Curt Harris: I think this is primarily a conception and pregnancy effect but there is some passive expose in childhood. It took me 40 years before I could do these studies because most women didn't start smoking until the 2nd World War. Then they started smoking heavily at that time. However, during that period many women were also exposed to ionising radiation because that was how the doctors were monitoring the size of their babies. So both ionising radiation and cigarette smoking during pregnancy may have been involved.

Martin Bushell: I think it is clear that miRNAs are going to be involved in programming to some degree or other, but whether or not they are causal, or whether a carrier, I think that is going to be critical. And what is driving those changes? There is obviously going to be some degree of epigenetic change but whether or not they are being inherited in the milk from the mother, or whether or not it is in pregnancy itself will take some time to work out as it is very complex,

Frank Slack: I would recommend to anybody in nutrition that they should include miRNAs biomarker profiling in their studies. Let me illustrate this by discussing ageing studied in c-elegans. This is a useful model because you can synchronise their development and they have very short lifespans with easy genetic analyses. It turns out that they don't have DNA methylation, so you can eliminate that sort of epigenetic role. They do, of course, have miRNAs that have transcription factors. MiRNAs definitely influence the adult lifespan in c-elegans. If you knockout argonaute or dicer when the animals are already adults, you have effects on their ultimate lifespan. We can look at isogenic animals that come from the same mother - they are all twins essentially – but even in isogenic animals grown in identical environmental conditions on the same plates at the same temperature and in the same part of the incubator, there is a spread in the lifespan of those animals. Some of them will last a week, some of them will have 3 weeks: the median is 2 weeks. So we asked whether miRNA levels, measured at the beginning of adulthood, could predict which of those siblings would have a longer lifespan. In fact some miRNAs do act as predictors of future lifespan. Now Ann Brunet's lab has shown that there are RNA-mediated trans-generational effects of lifespan in c-elegans[107] which is dependent on the H3K4me3 methylation complex and considered as an epigenetic effect. We have found that the levels of miRNAs when the animal is only one quarter of the way through its lifespan, provides an indication of their future lifespan and dietary restriction to induce longevity operates through these mechanisms and critically involves miR-71 and miR-228[108]. if you knockout miR-71 you have a short lifespan; if you over-express miR-71 you have an extended lifespan. But the animals that express slightly more, maybe 25% more of that miRNA, have a 25% longer lifespan that the ones that express less of it, so yes, the stochasticity is in the expression of certain genes. Now in humans it is possible that there will be a miRNA or two that could function as equally useful biomarkers..

Gerhard Aihauld: As far as I know, among nematodes c-elegans is the only one in which miRNA have been discovered, the only organism which has lost the entomology related to DNA methylation. I don't know if it is a coincidence or not?

Frank Slack: Yes, my point wasn't necessarily that c-elegans is instructive for all of biology because certainly without DNA methylation it is not instructive for humans, but what it allows us to do is to eliminate one whole class of gene regulation from the picture. We did not have to mutate the DNA methylation in order to eliminate it: it was naturally mutated before we started the experiments.

Leif Groop: In type 2 diabetes you have 65 variants associated with the risk of diabetes but the effect size is very modest. It is only during our last two generations that the environment has changed and thereby helps to induce diabetes. But we then find that the risk factors are actually indicative of resistance to the development of diabetes under these environmental conditions. So we need to think of these genetic factors as often protective – not just risk factors of increased disease.

Curt Harris: In humans I think it is important now to look at the 97% of the genome that isn't protein coding that maybe playing a role in the function of the various non-coding genes. For the protein coding genes, I would look at the genes that are involved in the processing of miRNAs and other non-coding factors. I am focused on cancer and believe we have many new opportunities for making progress now

Philip James*:* Yes but the problem of cancer is so complex. It seems to me that we haven't remotely begun to tackle the astonishing differences in cancer rates across the globe and with environments and diets changing so fast we may be missing things unless we are pretty coherent in the way in which we document mechanisms of carcinogenesis. The current dilemma is that we have cancer epidemiologists from around the world who consider putative factors in their cohort studies and find practically nothing. They completely neglect mechanistically the extraordinary genetic variation between people in their responsiveness to environmental factors which means that they will totally miss crucial things unless they look mechanistically at the casual chain of carcinogenesis in these cohorts. The same is true in considering saturated fat and coronary heart disease – cohort studies rarely show any relationship because there is such a big difference in the genetically determined responsiveness of people in any society to a standard saturated fat intake – it is only when one looks at the mechanisms –as measured in this case as cholesterol in the blood that one then realises the profound significance of the pathway. You have just been describing, with your miRNA analyses, putative signals of causal pathways, and we need those in the cancer process. Otherwise we are simply asking stupid questions about diet and cancer outcomes.

Pier Paolo Pandolfi: You are absolutely correct: unless you link these two pathways, clear pathways which are drivers in the cancer process you are doing association studies without any mechanistic basis. I think that again miRNA opportunities are great if we know what they target. So again although I am convinced that biomarkers are distinct from pathways, I still think that there is tremendous merit in trying to understand if the biomarker is simply a biomarker, or if it also leads to a pathway of interest.

Roderick Dashwood: I think the flip side of this is that sometimes we know too many mechanisms. I think a classic case in point is that there are the green tea polyphenols where there are probably about 50 or more mechanistic targets that have been identified in cell culture models, and in almost every animal model green tea is a wonderful, superb chemo-preventive agent at the doses we might drink, 2% green tea or whatever. But then when you ask how robust epidemiological evidence is for the effects of tea then we obtain very fuzzy data. So one has to consider how good the human studies are in their design and when we are looking at mechanisms we always come back to the issue of dose. The plausibility of a mechanism and its quantitative importance is the challenge.

Philip James*:* The other problem, if you are talking about polyphenols etc. is that we need to recognise the dramatic reduction in the types of plant foods now being eaten in the Western world - in the United States in 1900 there were 200 types of cabbage - there are now only 3 in mass production! So the biodiversity has gone dramatically down - and there has been a selective growing of a few foods with selections based not on the protective quality of plants or indeed their vitamin content but on the ability of the plant to grow uniformly, look good etc. We really have no idea what we have been doing with our plant selection processes when we consider a problem such as cancer prevention. I have been talking to horticultural experts, and they had not even measured folate in vegetables to see if there is any change - they were until recently trying to take out systematically n-3 fatty acids for 40 years to improve the shelf-life of the plants and their food products. It is unbelievable what has been going on!

Ingemar Emberg: - I think we are going to have to retain the idea that inflammatory processes are also involved in cancer. So what is the link now between miRNAs, inflammation and chronic inflammation - of course relating also to diabetes and to obesity, but particularly to cancer. I would like to emphasise the Hanahan and Weinberg paradigm of

cancer published basically very correctly 30 years ago and summarised recently[109] but originally criticised by Vogelstein[110,111]. Hanahan and Weinberg emphasised the multiplicity of pathways for carcinogenesis – it is not the linear idea about the cancer process: I think it is only the physicists and mathematicians that think it is like this. Since their original ideas miRNAs have entered the field as has epigenetics progenitor stem cells, tumour microenvironments and chronic inflammation. So how good are the links between the subject of this meeting and chronic inflammation and food?

Conclusions

***Philip James**:* From the Organisers' point of view, we recognise that we were organising this meeting at a very early stage in the whole process of understanding and we have already seen practically every main speaker presenting unpublished data. So this field is at a stage of exceptional development and certainly for the nutritional world one on which nutritionists really need to focus. Thank you!

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