could be potential clinical applications for cytoplasmic replacement or ooplasmic transplantation; Carol Brenner who has done a lot of the molecular biology, microgenetics of this work, together with Jason Barret; and Henry Malter who has been involved in the last three or four years.

I would like to backtrack a little bit after Susan Lanzendorf's presentation and, first of all, look at all the different oocyte deficits that exist. The most important one is aneuploidy. Aneuploidy is extremely common in early human embryos and oocytes, is highly correlated with maternal age, as I will show you. It is the most common problem in our field.

Chromosome breakage is not that well-known, not that well studied but is also very common. I am not just thinking about the risk of transmitting of translocations but also about spontaneous chromosome breakage that occurs in oocytes and embryos.

Gene dysfunction is being studied, particularly now that tools are being made available.

But we have to keep in mind a couple of

things here. When we study these phenomena there are a couple of things that are important to know. First of all, there is no government funding. So, it is all paid out of the clinical work. Secondly, we can only study these phenomena in single cells because we have really only single cells available to us. Thirdly, genomic activation is delayed. But that, I mean the finding that the early human embryo is really an egg that is on automatic. It is not activated yet. Expression by the new genome hasn't occurred yet. In the human it is considered to occur between four to eight cell stages, three days after fertilization. This is important because when we talk about ooplasmic transplantation we truly try to affect the period that occurs before genomic activation.

Here is the correlation between aneuploidy and implantation. On the horizontal axis you see maternal age. This finding is pretty old now. This was based on doing fluorescence in situ hybridization in embryos, in embryos that were biopsied and the single cells taken out. This was done by Munne and coworkers many years ago now. At that time, they were only able to do two or three chromosome probes, molecular probes to assess

chromosome. So, the rate of aneuploidy is pretty clear and it seemed to us, and many others, that this correlation is so apparent that you couldn't do anything with ooplasm or cytoplasm because in the mature egg aneuploidy was already present, particularly correlated with maternal age, and that problem was so obvious that not much else could be done.

But a lot of data has been gathered since this. Particularly what has been done is to do embryo biopsy, take a cell out at the four to eight cell stage. If you look at the implantation rate here, in the green bars and, again, on the horizontal axis you see the maternal age here, you can see that implantation--which is defined as one embryo being transferred giving fetal heart beat, the implantation rate diminishes significantly with maternal age.

What you see in the orange bars is what happens or will happen if one does aneuploidy testing. It shows that in the older age groups you will get an increase in implantation because embryos that are affected by aneuploidy are now selected out. They have been diagnosed. You can take those triploid or trisomic or monosomic

embryos out and put them aside so that you only transfer diploid embryos.

The thing though is that this is not a straight line. What we had really hoped is that we would have a very high rate of success regardless of age per embryo. That is not the case. If you use egg donors and you put embryos back in women of advanced maternal age, you will find that this is a straight line. So, if you use eggs and embryos that come from eggs from donors that are younger than 31, younger than 30 you will find that the recipient now behaves like a young woman.

So, what is different here is that it is not just the aneuploidy that is causing this difference, but also there is this huge discrepancy still that must be related to other causes, other anomalies that are present in the egg and, therefore, in the embryo that should be studied.

So, the question, and the question is raised very well by FDA, is there evidence of an ooplasmic deficit? Dr. Lanzendorf mentioned already fragments. These are blebs that are produced by the embryos. Both Jonathan Van Blerkom and our group have described a number of different types of fragmentation that have probably different

origins and causes.

The lower panel basically shows what you see in the upper panel but now the fragments are highlighted. These fragments in this case, here, occur at a relatively low incidence but you can score this. Trained embryologists are able to score this quite well, and proficiency tests have to be in place to make sure that this is done reliably.

There are different fragmentation types. Some of them are benign and some of them are detrimental. All depend on the type of fragmentation and the amount of fragments that are present. There are some as well that may not be cytoplasmic in origin, for example, there is multinucleation that can occur in cells of early embryos. All these are scored by embryologists.

If we look at this fragmentation phenomenon, here, again, on the horizontal axis you see how many fragments there are in an embryo and that is scored from zero to 100. One hundred means that there is not a single cell left; all the cells are now fragmented. Zero means there is not a single fragment that is seen. Then, there are scores in between.

Clinically, we know that you can get fragmentation up to 40 percent, like here, and you can still get maybe an occasional embryo that is viable but all the viability is here, on the left. When we looked at gene expression in spare embryos that are normal; they have been put aside and patients have consented to this research, when we look in these embryos, we are finding now that certain genes are highly correlated with these morphologic phenomena and are related to the number of transcripts of certain genes that are present in the cytoplasm of the oocyte and are present in the cytoplasm of the early embryo.

You can see here, in this particular gene, there is a very clear correlation and a very badly, morphologically poor embryo is here, on the right, have more transcripts of this gene in the cells.

There were a couple of genes that were looked at. Here is another one that is correlated in a different way which fits probably in the hypothesis that fragmentation doesn't have a single course. It shows though that there is a clear basis, at least looking at fragmentation, that this goes back to the egg and that the problems are present in the oocyte.

Another gene that has been studied for many years now by Dr. Warner, in Boston, is the gene that she called the pre-implantation development gene. This gene phenotypically shows high correlation with speed of development of early embryos. When we looked in the human we could basically--and this is very well known, you can see all these different speeds of development, development stages when you look at static times.

In our data base we separated patients that had different developmental stages where embryos may be eight-cell at one point and where sibling embryos would be seven cells or four cells. We took all those patients separately and we found 1360 patients that had very uniform rates of development. You can see here if we look at fetal heart beat projected from single embryos that there is a highly significant difference in implantation rate.

Similar to the model in the mouse, in the mouse you have fast embryos and you have slow embryos. The fast embryos implant at a very high frequency and the slow embryos can implant, it is not an absolute phenomenon, but they implant at a much lower frequency. This is under the control of

ooplasm, like in the mouse.

In the mouse the gene product is the Qa-2 protein and if it binds to the membrane the embryos will become fast embryos and you get good development, and if the protein is absent you get slow embryos, but you can get implantation but at a lower frequency.

Other cytoplasmic factors have been looked at. Transports have been looked at and now, with the availability of microarrays and other technologies, we hope that even though we are only using single cells for these analyses that we can correlate some of the expressions of these genes with viability of the embryo.

Here is an example. This is Mad2, which is a spindle regulation factor. We have looked at Mad2 and Bob1 and we have found--I apologize for the graph, it is pretty unclear, but the maternal age is again on the horizontal axis and younger women who had many transcripts present, a significantly lower number in all the women. Again, this was measured in the cytoplasm.

For this meeting, for the purpose of studying ooplasmic transplantation, is the issue of mitochondria genes. We have been interested in

this for quite a long time. Mitochondrial genome is, and I am sure Dr. Shoubridge will talk about this later in great detail, is a relatively simple conserved genome, 37 genes. On the top of it, at least in this picture, there is an area that has high rates of polymorphisms, the hypervariable area. Adjacent to it is the replication control region.

We have looked at oocytes, in the yellow bars, and embryos, in the orange bars, and compared mitochondrial DNA rearrangements. I have to mention that these are not potentially normal materials because these cells are derived from eggs that do not fertilize or from eggs that do not mature or abnormally fertilize, and embryos that develop so abnormally that they cannot be frozen or transferred. So, this is all from spare material. For obvious reasons, it is very hard to obtain appropriate control groups for some of these studies.

We found 23 novel rearrangements, and the frequency rate was astoundingly high. So, mitochondrial DNA rearrangements occur very frequently in oocytes; significantly less frequently in embryos. It has been postulated that

it is very likely that there is a block in place that selects abnormal mitochondria in a way that the corresponding cell doesn't continue to develop. You can see that fertilization block here. The spare embryos have less rearrangements than the oocytes, suggesting that there is a bottleneck, a sieve in place.

We have also looked at single base pair mutation at 414 logs. This was a publication from Sherver in, I think, 1999, who showed, and I am sure the mitochondria experts here may not necessarily agree with that work, but showed that in the natural population this mutation had a high correlation with aging.

So, we were interested to look at this. It was quite simple to study, to look at this particular mutation in spare human egg and embryo material, again, with the purpose of identifying cytoplasmic factors that were involved in the formation of a healthy embryo. We found that this single base pair mutation was fairly frequently present in human oocytes that were derived from women that were older, 37 to 42 years of age, and significantly less present in women that were younger.

So, when we look at the clinical rationale, there is a knowledge base but it is not necessarily specific for ooplasmic defects. Of course, we know very little about ooplasmic defects. So, a rationale for studying potential treatments for each defect does not exist.

The question is, and this came up actually earlier this morning, is there a rationale at all to do ooplasmic transplantation? Well, that is saying that all ooplasms are the same. Well, they are not. They are all different. So, I think that is the rationale. Not all levels of transcripts, not all proteins and not all mitochondria are the same in the ooplasm of different eggs.

What animal experimentation has been done, particularly with the interest of cytoplasmic transplantation? There is a whole body of research, and a lot of this work was done not keeping in mind that there was an interest in doing ooplasmic transplantation clinically, and I think Jonathan Van Blerkom said that. This work was done because there were other issues that needed to be studied, genetic interest in early development.

One of the papers not mentioned before is some interesting work done by Muggleton-Harris in

England, in the '80s, and they looked at mice that had what is called a two-cell block. These are mice that when you culture oocytes, zygotes in vitro, the embryos will arrest. You can change the environment but they will not develop further. By taking two-cell embryos from other strains of mice that do not have this two-cell block, it was possible by transferring cytoplasm to move the embryos that were blocked through the block. I think that has been a pretty good model for this work. However, this was done, of course, after fertilization and certainly is something that could be considered.

Many cytoplasmic replacement studies have been done from the early '80s onwards, particularly Azim Surani's group who looked at many different kinds of combinations of cytoplasm and cells with and without enucleation, different sizes, different techniques. Cytoplasm transfer has been studied in the mouse and in the monkey, and I will mention the work of Larry Smith, in Quebec, in Canada, who has created hundreds of mice from experiments that are very similar to the cytoplasmic transplantation model in the human. That work was done in 1992 and is continuing, hundreds of mice over many different

generations.

Then there is in vitro work done originally by Doug Waldenson, in Atlanta, and his work involves mixing mitochondria of different origins in the same cell and then studying cell function.

In Larry Smith's lab in Quebec, heteroplasmic mice have been produced, as I said. These are healthy, normal mice from karyoplasm and cytoplasm transfer. Karyoplasm is part of the cell that contains a nucleus and contains a membrane. Cytoplasm is also part of a cell that is surrounded by a membrane. They combined these in many different ways between inbred mouse strains with differing mitochondrial backgrounds because they are interested, like many others, in mitochondrial inheritance. Many of these animals have been produced over 15 generations apparently without developmental type problems.

We did an experiment in 1995-95. It was published in 1996 by Levron and coworkers where we looked at cytoplasmic transfer in mouse zygotes and mouse eggs, using F1 hybrids. We did many different kinds of combinations and found that in most combinations it did not really affect

development except when very large amounts of cytoplasm were fused back into the recipient cells. We found in one scenario a significantly improved situation where zygote and egg cytoplasm was combined.

The hybrid experiments have been done, which I mentioned before, for creation of cell hybrids with disparate nuclei and mitochondrial makeup. It has been done across species and across genes even. Normal mitochondrial function has been obtained in many scenarios. The only scenarios that in hybrids, as well as in mouse cytoplasm, karyoplasm studies that are not potentially normal have always been obtained across species or subspecies. Of course, those experiments are not really models for mixing mitochondria of two completely outbred individuals.

We have done work in the last few years that is similar to that of Larry Smith's laboratory but with the aim of looking at the mice in more detail and to see how fertile they are, for instance. So, here we take a zygote from one F1 hybrid and then mix the karyoplasm containing the zygote nuclei with the cytoplasm of another zygote.

It is a pretty small group here, 12 mice,

F1 hybrids. In those there were no apparent problems. The first generation is now 30 months old. We have done one more generation of 13 individuals that we just keep around to look at and until now there have been no apparent problems.

One of the problems with cytoplasmic transfer work, the ooplasmic transportation work in the human is the use of ICSI, intercytoplasmic sperm injection. It is basically taking a very sharp needle and go into the membrane of the oocyte. That has not been easy in animals, believe it or not, but it works well in the human, very well. The human egg is very forgiving but it doesn't work well at all in other species. In the mouse it has taken a couple of tricks to make it work, and that has only happened in the last few years. So, we think that we have a better model tentatively to compare what is done in the human, and to do this in the mouse. I am not saying that the mouse is the best model for these studies but it has all sorts of advantages. It is genetically incredibly well studied. It has a very fast reproductive cycle, etc. Here you see some embryos that have a good survival rate, 90 percent or better, from these experiments.

So, what is the clinical experience? The first time we approached the internal review board at St. Barnabas was sometime in 1995. The first experimental clinical procedures were done in 1996. When first results were obtained and also when we found the first indication of benign heteroplasmy and this was in placenta and in fetal cord blood of two of the babies, we reported this to the IRB and, of course, had to inform our patients. I think the question came up before, do you tell your patients about heteroplasmy? Well, you can only tell them about it when you find it. So, it was only found in 1999, and this is from this time onwards when it was incorporated in the consent procedure.

Then last year, after a rash of bad publicity, we went back to the internal review board but this was also at the time that the FDA sent us a letter. So, this second review is basically not going forward because we were asked to hold off until further resolution.

How do we do this clinical? Well, we made the choice to go for the mature oocyte and not the immature oocyte. We made the choice for the mature oocyte because there is incredible experience with IVF as well as intercytoplasmic sperm injection

manipulating these eggs. These are small cells that are genetically similar to the egg and these can be removed microsurgically. There is experience with injecting sperm from male factor infertility patients. Forty percent of our patients have male factor infertility, possibly more. So, there are more than 100,000 babies born worldwide from this ICSI procedure.

So, we felt that what was a better approach possibly than using the more classical micromanipulation procedures that involve, for instance, the formation of cytoblasts and karyoblasts and then fusion, which we thought was maybe just a little too much. So, we took cytoplasmic transfer using ICSI as a model. There are advantages to that and disadvantages. You could do this also at the time the zygote is formed and the two-cell is formed. This has been a clinical pilot experiment we chose. For the first lot of patients we chose the mature egg.

The procedure was already shown by Dr. Lanzendorf but basically you pick up a sperm and then go into the donor egg. I would like to point out here that the polar body, right next to it--the human egg is very asymmetric. It is polarized, and

the spindle that obviously under light microscopy and also in this cartoon is not visible, is located very close to the polar body. So, the idea is that we should not transfer chromosomes from the polar body. Therefore, we keep the polar body as far as possible away from the area where we select our cytoplasm from. Then, when cytoplasm has been absorbed in the needle, it is immediately deposited into a recipient egg.

Pictures don't tell you very much because they are static, but here is the sperm cell and then going into the donor egg, here is the donor egg. The polar body cytoplasm of the sperm is now here, and then is deposited into a mature recipient egg. When we do this we make videos so that we can see that cytoplasm has been transferred, but also in the usual circumstances the cytoplasm between oocytes is very different, has a different consistency, different refraction and, therefore, you can usually immediately see the amount that is transferred and injected, and that is highlighted here.

We have done 28 patients so far. Five had repeated cycles. three of those became pregnant and had a baby the first time and challenged their

luck and came back again. They were all egg donation candidates.

Now, I need to say something about this. First of all, there are a lot more patients that want to be candidates but our feeling and also we agreed that we should do these patients in-house because there are tremendous differences in outcomes, clinical outcomes between programs. So, if a patient would come that has ten failed cycles elsewhere, it is not at all unlikely that she could become pregnant in our program or in another program if she switched programs because laboratory procedures and clinical procedures are very different from program to program. So, we felt that at least there should be a couple of cycles done by our own program if the patient came from elsewhere.

The average number of previous cycles in these patients is well over four. These patients have recurrent implantation failure. So, they come in. They do not become pregnant. We put multiple embryos back. They have a good response to follicular stimulation so they make a lot of eggs but they do not become pregnant. They have normal fertilization rates. They also all had recurrent

poor embryo morphology. However, there was one exception to that. There was one patient that had normal fertilization but zygote block. The zygotes basically fall apart in fragments and other zygotes would never even do that. They would just stay. Fertilized as they are, they would never divide. So, one of the 28 patients did not have poor embryo morphology. She simply did not have developing embryos.

A number of these patients were male factor patients and it is important to realize that when you get poor embryo development, some of that may be caused by the male factor. The sperm may be the cause of abnormal development, particularly because the sperm brings in the centriole that is obviously crucial for division. The centriole in the human is inherited through the maternal line. It is possible, and being suggested by Jonathan Van Blerkom that men that have abnormal centriole function. Certainly, we have found that in some subsets of men there are high rates of mosaicism, indicating that there are problems with division and, therefore, their infertility is correlated with embryonic failure.

When I say nine male factors, it really

means that they had abnormal semen. There could have been other male factors as well with normal semen. There can be patients that have normal sperm but they can still be infertile. Five of these patients had repeated miscarriages. So, five of them had been implanted before but always miscarried.

So, we did 33 attempts. Two did not have viable embryos for transfer; 21 transfers and 13 clinical pregnancies. There were more clinical pregnancies from this patient group, and the reason for that is that in order to do the cytoplasmic transfer we only used ten percent or so of the cytoplasm of a donor egg. So, we actually use donor eggs several times. We go into the same donor egg of two times. Twice. We go in there twice, and sometimes more if only a few donor eggs are availability. Most donors are good stimulators so they will have a good response to follicular stimulation and will make a lot of eggs. So, the procedure yields a lot of eggs that are not used. What we offer to our patients is that those eggs are injected by sperm from the male partner and that embryos resulting from this are frozen for later use. So, it is not only cytoplasmic transfer

procedure, it is also an egg donation cycle. There are patients that don't come back for another attempt of ooplasmic transplantation or they are discouraged to do that, and then they come back for frozen embryos from the donor eggs that were injected with the sperm from the husbands.

So, the data I am showing here is clean data. These are pregnancies that occurred from transferring embryos that were derived from ooplasmic transplantation. But if the patients have failed, some of them may have another chance using the frozen embryos.

There was a firs trimester miscarriage. There was an XO pregnancy. Obviously, these are fairly common, the single most common chromosomal anomaly in early pregnancy. This happened at the end of '98. A few months later we had a twin pregnancy and one of the fetuses on amnio was diagnosed as XO as well. We published this a few years ago.

With that information, we returned to the internal review board to let other patients that are undergoing this experimental protocol know that this may be a potential issue. If you look at the statistics, many statisticians have told me that

there may be an issue or there may not be an issue.

On twin was born and also a quadruplet was born. This is one of two patients where there was a very clear improvement in embryo morphology. However, we understand that we are so biased as embryologists that maybe we were imagining some of this. So, four embryos were transferred. Of course, in that respect we should have only transferred two or so. This was a patient who had five previous attempts and always had very poor embryos and now, suddenly, the embryos looked much better. In spite of our advice in the consent form that were given to her at the time of these products, she did not elect the selective reduction.

Seventeen babies were born. Pediatric follow-up has been done only in a proportion of them that we know of. By that, I mean some of these patients are from abroad. The issue came up before that really not all these patients are interested in follow-up by us, and we have tried to be quite forceful with them. So, we have been able to do follow-up in 13 of the 17 babies. However, more recently it is more likely that some of them will refuse further investigations by us. This is

not just this particular group. That is common for all infertility follow-up, that you lose sight of these patients. Some of them will move and not even leave a return address.

On twin, this one twin that was born with mixed sex, a boy and a girl, the boy at 18 months was reported to have been diagnosed by pervasive developmental disorder, not of specific origin. The incidence of this in the recent literature is 1/250 to 1/500. This was reported to us in June of last year. That was at the age of 18 months, and we have no good follow-up. This is just what is going on with this little boy.

One issue that comes up is, well, does this really work? One way of investigating this is to look at attempt numbers. You see here, on the left--the colors are very confusing but on the left you see the first attempt number here in the general IVF population that we studied. The second attempt number, the third, fourth, fifth, based on about 2500 patients. So, you can see in the first attempt number the procedure rate. In the first attempt number the success rate is very high but then it significantly drops, which makes sense because it left us with a more complex population.

The third attempt is also significant because of the high numbers involved. Then it sort of flattens off.

If we look at the per embryo, it is also marked. This is the incidence of success by embryo. It is well over 30 percent when you come the first time, then it significantly drops to less than 20 percent the second time, and again drops significantly the third time to about 15 percent, 14 percent.

Now, the ooplasmic transfer cases are here, in the red bars, and they have an average of about 4.8 previous attempts. So, they actually are between these two bars. That is where they should be. But these patients also contain patients that have repeated failure with apparently normal looking embryos. So, you can't really make that comparison strictly but it is suggestive that at least it worked to some extent. This is early days, only 28 patients and 33 cycles.

Some comments about the mitochondria work that we have done. Spare eggs can be looked at and you can use a stain for mitochondria and then look if the egg fertilizes where these mitochondria go to. We found in a number of cases they can go to

the blastomeres, sometimes not all blastomeres but it is well proportioned. There was one indication that they can survive for at least a few days, but the best was to look at the polymorphisms in the hypervariable area of the mitochondrial genome. We did that work originally with regular sequencing. Se looked at spare eggs and embryos that were not transferred after ooplasmic transplantation.

Then we looked at amniocentesis. That was actually quite frustrating because it is not easy to get good cells there for this type of work. In a couple of these babies we have been able to look at the time of delivery and obtained placental tissue by being present at delivery, and also obtained fetal cord blood.

If you look at the incidence of heteroplasmy, and I must say this again, this is heteroplasmy in the hypervariable area, maybe we should distinguish that from other forms of heteroplasmy because these are extremely common in the general population. Spare embryos, about half of them, after a few days of culture, showed these polymorphisms so that you can basically confirm that mitochondria were present from the donor.

On amniocentesis we did only ten, and

three of them sere positive. So, mitochondria were present from the donor in amniotic cells. In the placenta it was 3/13. We are only looking to obtain blood at the first year from those babies that were positive at the time of delivery, and two have been tested thus far and are still positive for donor mitochondria.

Recent work by Carol Brenner--because this is done by sequencing which, I think most agree, is not that sensitive a method--recent work by Carol Brenner has shown using molecular beacon for hypervariable locations, using this work it has been found that up to as much as 50 percent of the mitochondria in the blood at the time of birth would be positive for the donor. So, when we inject 10 percent, it certainly doesn't mean that there will always be 10 percent, but no doubt in some children there will likely be a trend to homoplasmy and in others maybe a consistent heteroplasmy.

The word heritable was used this morning by Dr. Hursh, and I do object to that because there is no proof at all that this is heritable, but it is certainly possible. No proof so far.

Here are the three famous words, germline

genetic modification, used by J.C. Barritt in the publication last year. There were four authors on this paper. The three other authors do not agree with this wording. So, it only appeared in an abstract; it didn't appear in the regular text. We don't agree because we don't think that it is modification. It is a kind of difference, change, or maybe I don't have the right word. It is different from what has ever happened but in my opinion it is not germline genetic modification.

Mitochondrial diversity, not all that dissimilar from this, occurs in the hypervariable area in 10-15 percent of normal humans. This is recent work from more sensitive assays by Tully and coworkers. I must reiterate that the hypervariable area is a non-coding region.

One issue that hasn't come up today yet is that maybe this is a technique that places at risk the transfer of mitochondrial disease. Mitochondria are maternally inherited so in egg donation you have mitochondria 100 percent from the donor. There are no known cases of mitochondrial disease after egg donation. Certainly, when you use ten percent of the mitochondria from the donor, would there then be suddenly an indication that

there is an increased risk factor of mitochondrial disease?

I will quickly go through the risks, the potential risk factors. Mechanical damage has been raised as a risk factor. While it is an ICSI derived procedure, the survival rate with this procedure was better than 90 percent. However, it is slightly higher, in our lab at least, than the average damage rate to eggs after ICSI just injecting a sperm.

Cytoplasmic transfer, the fertilization rate is over 65 percent. So, we think that is a normal fertilization rate. With ICSI there have been 100,000 babies born. The pre-implantation development with ICSI seems like IVF and the malformation rate seems like IVF. Certainly, with the bare minimum results we have, we think that the malformation rate from our procedure also resembles that of IVF.

So, what are other risks potentially to offspring? Inadvertent transfer has been raised as a potential issue. If you have unique organelles you don't want to transfer those and boost them. Like the centriole sperm derived, centriole is separately placed in the cytoplasm. The sperm is

intactly placed in the cytoplasm. So, it is unlikely you will lose that.

Avoid the spindle, and if you cannot avoid it there should be cytokinetic analysis. So, one thing we do is every egg, every donor egg from which cytoplasm has been taken, we give it to the cytogeneticist that is specialized in single-cell cytogenetics to confirm that the chromosomes are still there. In two cases we couldn't confirm this in two eggs and the next day we, indeed, saw things that we call subnuclei. These are basically small nuclei that were present in the periphery of the egg, not in the middle but in the periphery, confirming that the cytogeneticist was right. So, it is a good thing to have a cytogeneticist around, otherwise one should do very detailed study of the zygote, or one could use a microscope that will visualize the spindle at the time of piercing, as Dr. Lanzendorf has done.

Enhanced survival has been raised as a potential risk to the offspring. The embryo is now better and, therefore, you will get higher implantation rates and implantation of embryos that would have normally, under normal IVF/ICSI conditions not have been implanted.

Aneuploidy is common. Aneuploidy is the issue that has raised a lot of concern in this particular group of patients. Aneuploidy is common. It has been found that this is enhanced in ICSI by one percent, more or less one percent. The most common anomaly that is found in ICSI and also in the natural population is XO. This is exactly what we found in two patients that this in early pregnancy.

Then heteroplasmy, is that a risk to the offspring? Well, we have confirmed three polymorphisms in three births. We think that these are common in the population, or similar polymorphisms are common in the population. In general though, heteroplasmy is very common in early human embryos when we studied this experimentally in the spare material. From the animal experimentation, there is no evidence of risk between outbred individuals in the same species. There are clearly anomalies that have been shown in the literature when you don't use the same species, or when you use highly inbred individuals of the same species.

What are the risks to the mother? An elevated incidence of chromosomal anomaly should be

considered a risk, if there is such a thing. There is no statistical evidence for this so far. As I said, aneuploidy is extremely common, and XO is the most common form.

What cell issues can there be? Should there be donor screening? If we do that for this procedure, I don't know the complexity of that. I don't know the cost factors associated with it.

Abnormal zygotes, fertilized eggs, I used the mitochondria from there to inject back into another zygote. But that has been done. It has been reported by a group in Taiwan. I think this was raised here before, can you maybe look at other cells and get mitochondria from other cells? That has been done as well. If I have a little bit of time later, I will get back to that. Actually, there has ben one abstract, where mitochondria were taken from granulosis cells, the cells that surround oocytes. These were then injected into the patient's eggs.

We videotape the whole procedure for later evidence that we transferred the cytoplasm. Can one use frozen oocytes? We have not used frozen oocytes. The disadvantage of our procedure is that you have to simultaneously stimulate and monitor

the patient and the recipient and retrieval and maturation of the egg has to occur on the same day. That is not simple. That is actually quite a challenge. So, using frozen oocytes would be an advantage but oocyte freezing by itself is an experiment we feel, therefore, we stayed away from this.

We do the chromosome screen of the eggs that are used. Of course, before you transfer the embryo you could also do another chromosome screen. We have stayed away from that but we have that technology because these embryos are often not well formed, and are already challenged by the procedure and taking another cell out of the embryo before it is transferred may be detrimental in this particular group of embryos, not necessarily in other groups of embryos.

So, what further non-clinical experimentation should be done? Well, we should look at costs. I am not sure that the primate model is a good model for human reproduction but others probably dispute that. The mouse model we are using. Although there are profound genetic and profound differences with the human, that is more affordable and results are very rapidly obtained.

The issue with ooplasmic transplantation and the way we have done it and Dr. Lanzendorf's group has done it is that that is just one particular application. There is a host of applications that are waiting that, in one way or another, involve ooplasmic transplantation, not necessarily for the same purpose as I have described here. One of them is treating mitochondrial disease. You could replace the whole cytoplasm or ooplasm of a donor egg in a patient that is at risk of transferring mitochondrial disease to offspring. That is one potential application.

There are other applications as well, avoiding aneuploidy by going into very immature eggs and changing the regulation of how miosis occurs by trying to maintain regular ploidy rather than aneuploidy. It is obviously under cytoplasmic control. So, if you were to do this early, at least in theory we believe you could avoid aneuploidy. That would be important particularly since aneuploidy is the biggest problem area in our field. There are other applications as well.

Here are the two babies that had benign heteroplasmy. This picture was taken two years ago

so they are almost four years old and they are both doing fine.

Finally, just a few words about transferring mitochondria, this was reported in an abstract last year. This was shortly after September 11 so I was waiting in the room for that particular presentation but they never came to the country and this meeting was very poorly attended because this was only a few weeks after September 11. Anyhow, the abstract argues that there is a single course for ooplasmic problems, and that is the mitochondria. There is absolutely no confirmation for that. Mitochondria obviously may have a higher rate of mutation but there is no proof that this is the only problem. They used somatic mitochondria which is an interesting idea, but the isolation process could be an issue, for instance formation of free radicals.

Age-related mutation should be considered since these are mitochondria from somatic cells and may have, or very likely will have age-related mutations. They are also replicating mitochondria. What will happen in the recipient cells? That is an interesting question that will come up. Mitochondria in eggs do not replicate. They do

that after implantation. So, they are actually somewhat dormant in that respect. Somatic mitochondria are very different. Somatic mitochondria have multiple mitochondrial genomes per mitochondrion for instance, whereas oocyte mitochondria only have one genome. So, they are very different although they seem similar.

That is all I have to present. Thank you.

Question and Answer

DR. SALOMON: Thank you very much, Dr. Cohen. Obviously with the changes in this morning's schedule we are not quite following the time line here but this is such an extraordinarily rich presentation in terms of questions that I think we are just going to have to spend some time to address these. I think this and Dr. Lanzendorf's are kind of pivotal. So, I do realize that we are not on time but we will deal with this in a little bit.

I have a lot of questions but let me just start with one little part and then turn it over to some of the others, as I am sure I won't be alone. You know, the one theme that we picked up in Dr. Lanzendorf's presentation is what is the basic science background for doing this? Then we will go

on to talk about what is the clinical evidence for doing this, and you have given us a lot to think about.

So, going back to the basic science evidence of it, you presented two kinds of basic science arguments for ooplasm transfer, i.e., kind of a rationale. One was this PED phenotype. The other was some data on Mad2 mRNA transcript numbers and maternal age. Again, it is okay if it is not convincing but I didn't find that either of those was clear to me or convincing.

With respect to the PED gene phenotype, I didn't understand how you related slow and fast embryos back to a PED gene phenotype, and then how that had anything to do with ooplasm transfer. Similarly, you implied that gene arrays and other technologies have shown differences in gene expression as a function of maternal age in terms of implantation failures, and that certainly makes sense to me in some of the functional genomics we do in angiogenic stem cells. But how do you relate a change in transcript numbers to transferring 10-15 percent of ooplasm? I mean, what evidence is there that 10-15 percent of ooplasm transfer provides an increase in, in your example, Mad2 mRNA

transcripts, and does that increase them to a level that is equal to more successful implantation phenotype? So, I guess those are the kinds of questions I would like you to address since those are your arguments.

DR. COHEN: I have a short memory so I will start with the last one, why ten percent? It seems so little. If it was a blood cell it would be little, but the human egg is the largest cell that exists. It is an enormous volume and it is known that you can lose 75 percent of the volume and still get a human. So, 75 percent of the volume can be destroyed and since you have to have some unique organelles like chromosomes and a centriole, it is likely that you can reduce that volume even further. So, ten percent is not little at all, and we have calculated it is about 10,000 mitochondria for instance. So, it is a huge amount. That is considerably higher than the number of mitochondria in mouse eggs for instance that are smaller.

So, coming back to the PED, I think what is different in other developmental sequences is that in mammalian fertilization early development the embryonic genome is not active yet. It is all

dependent on what is present in the egg. So, when you sequentially look at a transcript like actin and you look at it one day and the next day and the next day, you will see it diminished to levels that you could almost call starving, if that would be the right word for it, but it really dramatically diminishes and then at the activation of the genome the embryo starts taking care of all this and you can see that going up.

So, what this shows is that these levels of expression are so different between cells of the same stage that it is maybe not direct evidence but it is likely that there is a physiological difference between these individuals. I think Mad2 is very likely because there it is a spindle regulating factor and it is related to maternal age, and we know that in maternal age not only is there an increase in aneuploidy but the typical non-disjunction form of aneuploidy in mosaicism is also related to maternal age in the human in early development. So, I think it is very plausible.

In PED, in the mouse at least, a human homolog has never been found. I was just indicating that there is a phenotypic similarity. We are looking for human homologs and they are

probably in the HLA system.

DR. SALOMON: But I am just pointing out to you that to make your case what you need to do is show us that if you transmitted 10-15 percent of the ooplasm that therein would be contained enough messenger RNA from Mad2 to alter the transcriptorsome of the recipient in such a way that at least you wouldn't have to demonstrate in the first set of experiments that it was functional, but just demonstrate that even numerically the transcriptorsome would be altered significantly enough to bring it into a range. Then, of course, the next set of experiments would be to show that it is functional.

DR. COHEN: Would you give me permission to do this in the human?

DR. SALOMON: We will get back to that, but I think what we are all trying to do is respectfully sit here and say, okay, what is the data? What is the data basic? What is the data in animal studies and what is the data in clinical? I was just starting with the basic. You have made a very intelligent start by saying, okay, look, here are changes in transcriptosome, changes in messenger RNA levels. My response is, okay, you

know, I am following you but I am saying it is not convincing. I mean, you have to give us a little bit more to justify this at this basic level. If the data is not there, the data is not there.

DR. VAN BLERKOM: Just to clarify something, you are not saying that the embryo from fertilization to, let's say, the four-cell stage is transcriptionally inactive, are you?

DR. COHEN: No, it is not. There is some leakage, yes.

DR. VAN BLERKOM: Because, in fact, things like actin, etc. are made off maternal--

DR. COHEN: Sure.

DR. VAN BLERKOM: Even in the mouse where it had been earlier thought that major genome activation occurred around the two-cell stage, in fact it has been brought back earlier to the pronuclear stage. In fact, there is probably embryonic genomic activation very early, but the major genomic activation, that is the major switch from the maternal stores to a whole embryonic program is probably at about the four- to eight-cell stage, but it is not transcriptionally inactive.

DR. COHEN: Yes, thank you for explaining.

DR. NAVIAUX: How long would you expect to be able to detect transferred RNA in the embryo? What is the half-life?

DR. COHEN: The half-life is very short I think.

DR. NAVIAUX: Would you expect it to be equivalent to the RNA already in the oocyte?

DR. COHEN: The experiment that hasn't been done is to take an oocyte and then take one of the two-cell blastomeres and then take one of the other cells of the two-cell blastomere and look sequentially like that. It is done by indirect, by looking at populations and then comparing the different stages. It is very clear that it diminishes from stage to stage. It is very sensitive. It diminishes very rapidly.

DR. NAVIAUX: I was trying to get a feel for the window of opportunity for other potential genetic events to occur from the transferred nucleic acid, including potentially the retrotransposition of this.

DR. COHEN: I have no evidence for that. It is certainly possible.

DR. SALOMON: Dr. Sausville, Dr. Mulligan and Dr. Van Blerkom.

DR. SAUSVILLE: The concern I have about the direction of the conversation that is happening now and, again, I congratulate you on a very thoughtful presentation but I think it does highlight one of the issues, that mitochondria have been put on the table as one explanation for a benefit. I guess we are going to hear more about mitochondrial physiology in which, hopefully, there will be some clear and direct evidence that mitochondria might do such a thing.

But we have just heard of another class of molecules, your presentation brought up a particular class of mRNAs, forgetting the whole issue of mRNA in general. I mean, this points to a key difficulty that I think we have in thinking about this in that one of the components of an IND is actually a definition of what actually is the substance under investigation in an IND. I am a little concerned, even if one believes there is an effect and we heard earlier this morning that there really isn't any evidence that there is an effect, is how we would define the potential basis for investigational activity with this. Are we going to have ooplasm that has a particular type of mRNA or a particular number of mitochondria or a

particular class of mitochondrial genomes? I would be interested in your thoughts on how one would define, in essence, the focus of the IND application in this regard.

DR. COHEN: I asked that question to the FDA representatives a few months ago and I didn't get an answer because I don't think they understand that either.

DR. SALOMON: I think that is why we are here.

DR. COHEN: Yes, so I wouldn't know how to do this. I have no idea.

DR. SAUSVILLE: Well, if you don't--

DR. COHEN: Personally, I have not experienced this IND process. Looking at the IND process, it is so different, the psychology of it is so different from this type of typical medical intervention approach that it is extremely difficult to come up with a solution.

DR. SIEGEL: Just from a historical perspective, there are certainly plenty of precedents in biological development in particular for products whose active ingredients are not well identified. Some of the earliest biologics, regulated as biologics, were horse antisera and,

you know anti-venoms and toxins and so forth. Of course, over the last couple of decades the field has moved to much more highly purified products which are, therefore, easier to ensure that you don't have unwanted materials and where you can quantitate what you have. We certainly support that area of development, but there is nothing about an IND process per se that requires that you have a handle on what component it is of what you are testing that is the potential active component.

DR. SAUSVILLE: Ah. But, on the other hand, my understanding is--and those are good examples actually--that despite that lack of definition there is, nonetheless, a very precise assay that will tell you that your material is functioning as you think it is functioning. Correct?

DR. SIEGEL: That is right, and we certainly require by the time of licensure a potency assay. That is required by regulation and that requires development of information. In fact, it is the case for many that we now have under IND. However, the development of the potency assay often occurs concurrent with the early clinical studies because it requires identification of markers that

can be measured that, hopefully, then can be validated to be predictive of the desired clinical effect.

DR. SAUSVILLE: So, that then actually does play back to the question I asked. You pointed to the limitations appropriately of the animal models that are around for this type of work. Nonetheless, it would seem that such models might be the place to begin to develop this type of information that could be a basis for conveying confidence at the very least, forgetting the IND process, that you would be able to advise a particular patient that the procedures that are in place are likely to be productive of some normative standard of activity through the process.

DR. COHEN: Yes, and I think that the body of literature is not enormous, but particularly the work of Larry Smith is very convincing and this is done in outbred mice going through 15 generations with apparently normal development, normal growth. What else are you looking for?

DR. SAUSVILLE: I would like to know what conveys that normal growth. What is the physical basis of that normal growth?

DR. COHEN: That is more than a textbook.

I mean, that is the whole field of early embryology. You are looking at an extremely difficult process that is hindered by all sorts of factors in terms of how we can study it. I am as curious as you are. So, I appreciate the concern, but that is looking at the oocyte like a product; let's understand the product, and I think what is being attempted here is to take something this complicated and then put it in the form of IND. I have no idea how to do that.

DR. SALOMON: I think we will return to that this afternoon. I think the issue that has ben well articulated now is what--I mean, we can always take every one of these questions and get down to these really big, fundamental scientific questions and we all know around the table that you are not going to know every single thing about how you create a normal embryo before you do these studies. No one is holding you to that sort of a standard. But it will be really interesting to talk about what it is we want to know, and what kind of scientific questions will be answered even while perhaps certain clinical studies are going on just to make sure that there is development along the right lines in the field. Dr. Mulligan?

DR. MULLIGAN: Can you give us a sense of how you test for fragmentation of either mitochondrial DNA or nuclear DNA and then transfer? In principle, if you such out the cytoplasm there is some chance for fragmentation of both of those DNAs, and it would be, I think, very important to see if that does occur because once you have kind of disrupted the normal mitochondrial architecture it is like doing gene transfer, that is, it is like injecting fragments of DNA and there is every expectation that there would be uptake by the chromosomal DNA like normally occurs. So, have you looked at ways in a single cell?

DR. COHEN: I would be more concerned about it in the isolation process of mitochondria, but here is a package of cytoplasm that is moved from one cell to another cell within seconds in a synchronous fashion. So, I don't think that concern is really valid. It would certainly be valid I think in the work that was done by the Taiwanese where mitochondria were isolated and then processed in ways we don't know yet, but they were processed, isolated from granulosis cells and then injected into the recipient cells. I think there that is a concern because you do true isolation

process of an organelle. In our case we are transferring cytoplasm intact.

DR. MULLIGAN: Yes, but I thought you said there is a risk of actually getting contamination.

DR. COHEN: Sure.

DR. MULLIGAN: So, in principle that has the potential for fragmentation, and isn't that key to see whether or not there are detectable bits and pieces of genomic DNA?

DR. COHEN: No, we have just done classical cytogenetics. We looked for whole chromosomes; we have not looked for bits.

DR. MULLIGAN: You mentioned that you have a good cytogeneticist who can detect things, that is, the most gross assay for a microbiologist to be able to detect things much easier.

DR. COHEN: Yes.

DR. VAN BLERKOM: Maybe you could clear up some points on what you said. As I recall, in the initial births the amount of DNA that was detectable was a trace amount.

DR. COHEN: There was nothing in the original, right.

DR. VAN BLERKOM: Now you are saying that Carol has seen up to 50 percent. Was that from the

original samples using another assay, or is the mitochondrial DNA expanding?

DR. COHEN: No, they are all the same samples and, I am sorry, I just gave you the wrong answer because in the first births we were not able to confirm heteroplasmy; we found a homoplasmy condition. In the births since then, with regular sequencing, we found levels, we found levels up to 20 percent.

DR. VAN BLERKOM: At birth?

DR. COHEN: At birth. That includes the placenta. Placenta seems to be always higher. With the new method those same samples were reassayed and there we found levels up to 50 percent.

DR. VAN BLERKOM: So, it is very likely a sensitivity issue. So, you don't have evidence that there is an expansion of the mitochondria from birth.

DR. COHEN: No, I don't have evidence of it yet but I have always been interested in that.

DR. VAN BLERKOM: The other question then is if you look at the process of cytoplasm transfer, which I don't think is an issue related to mitochondrial damage just from the logistics of

the transfer process, in at attempt to standardize, and I know you have done this so maybe you should talk about the data where you have actually taken the same amount of cytoplasm from different portions of eggs and then counted the number of mitochondria, and there are differences.

DR. COHEN: Yes.

DR. VAN BLERKOM: So, maybe you can talk a little bit about the extent of differences that you get that is location dependent, and how that my reflect on what you are putting back, what you know and don't know about the magnitude of the donated mitochondria.

DR. COHEN: The procedure is standard, however, ooplasm differs from egg to egg. There are physical properties that are different. So, you want to pick up cytoplasm just using suction. It is certainly not comparable from one cell to the other. So, in some cases the procedure differs from other cases. Also, the cytoplasm is not sampled statically. I should have brought a videotape. It is actually sampled throughout the whole area opposite the polar body rather than one area. It is a good, valid point. It is known that the egg is very dissimilar from area to area so we

try to sample a relatively large area of the egg. We have also varied the amount of cytoplasm that we transfer. All I can say about that is that if you look at the higher amounts, the higher volumes of cytoplasm that has been transferred, the more likely it is that the procedure is unsuccessful, for reasons I don't understand but that is what the finding was.

DR. SCHON: Just a clarification, Dr. Mulligan, I am gathering that the question about fragmentation--I won't talk about the nuclear transposition events, but at least for the mitochondrial DNA transposition events, my guess is that, first, there would be very few fragmentation events to begin with. It is a tiny molecule. It is stuck in nucleoids inside the mitochondria. If you visualize what is going on, it probably wouldn't happen that frequently. Let's say it does, and it does go into the nucleus, first, the worry would not be whether that transfected DNA would actually do something because it has a different genetic code. Whether it would transpose into some other gene, it may but again the likely hood would be low because there are at least a thousand and maybe more nuclear embedded

pseudogenes of mitochondrial DNA to begin with so it would probably go in by homologous recombination into places that are genetically quiescent--I don't know how else to put it. So, it could happen but I wouldn't give a huge probability for it.

DR. MULLIGAN: Yes, I would think the risk would be cytoplasmic DNA actually integrating in the incorrect location. I would very much doubt that you would get what you say, homologous integration into pseudogenes or mitochondrial sequences. So, it would be the risk of insertions comparable to a retrovirus insertion. It is like thinking of injecting a plasmid DNA. I guess what I didn't know is what the chances that the intact mitochondria would actually, by whatever vortex when you are trying to suck out the cytoplasm, with there is damage such that you would actually get, you know, naked DNA. But the other half of it, of course, was the nuclear DNA which I think would be much more likely to have the same potential for integrating in some incorrect location. And, I think it is very, very tough from all we know with gene transfer to assess the efficiency of the process. It is very amazing how different approaches to gene transfer can dramatically give

you different efficiency. So, even several molecules, you know, if they are given by a fancy method like this, this could be the most efficient method we have relative to other systems.

DR. SCHON: Then, could I just comment to Dr. Sausville? I actually think that trying to figure out the exact active ingredient, if you will, of the ooplasm may well wind up being a bottomless pit. It is the ooplasm itself that may actually be doing it. There is not evidence that it is mitochondria. If you were to merely just put in mitochondria or some subfractionation element, you might get nothing also. I think there is so much synergism going on that merely doing pair-wise analyses, each alone might give no outcome whereas ooplasm, where we have no evidence that there is outcome yet, might give an outcome, and it should be borne in mind.

DR. RAO: Just a couple of clarifications for what you talked about. You made a point about saying you disagreed with germline transmission. Was that because it hasn't been tested in germinal cells or is it because you want to wait for F2? I mean, what is the reason?

DR. COHEN: Also the modification. It is

not a modification and it has not been proven to be heritable.

DR. RAO: So, because it is not heritable.

DR. COHEN: Not proven to be heritable.

DR. RAO: The second question was on the point that you made about somatic mitochondria, was this ooplasmic mitochondria, and you said one big difference was in the rate of cell division. But do you think there is any other major difference? The other point you made was about it is a multiple genome. Did you mean that it is because it had inherited mutations and that is why it was more than one genome?

DR. COHEN: Yes, it is all those things. There are multiple genomes and mitochondria from somatic cells, anywhere from two to ten I think. In eggs the ratio is very close to one. So, that is different. The other difference is that mitochondria and oocytes and embryos do not replicate, whereas somatic mitochondria do. So, that would be a different control situation. It is an interesting suggestion.

DR. RAO: The last question is that there seems to be a suggestion that there won't be a whole lot of mitochondrial transfer that would have

occurred, at least it was a surprising result that you had in mitochondrial transfer. What is the basis? I mean, I am not absolutely sure why people thought that you would not get mitochondrial transfer and maybe you can tell me.

DR. COHEN: Well, if I had this discussion several years ago it may have been a different story, but we use it as a marker. We are just interested to see what happened to these mitochondria, and this is the outcome of it. But it was the advantage of hindsight. You are totally right, I mean, it is not surprising.

DR. CASPER: I want to go back to Dr. Mulligan's point again. We do have some experience in creating mitochondrial preparations from granulosis cells, from mouse embryonic stem cells, from human umbilical cord blood to hematopoietic stem cells and also from human leukemia cell line, and it is actually quite easy to do it. There are some technical issues that took us a while to actually figure out, but morphologically at least when you look at the preparations they seem to be pretty pure, intact mitochondria. So, the actual morphology at least of the mitochondria looks normal. We have injected these mitochondrial

preparations into mouse oocytes and zygotes.

There is a stain of mice called FVB mice that have a mitochondrial defect and oocytes fragment in vitro, and with both granulosis cell, so somatic cell mitochondrial injections and with stem cell mitochondrial injections we have been able to prevent at least 50 percent of the fragmentation rate in those oocytes. We have also injected mitochondria into mouse zygotes and we have found that, contrary to there being any detrimental effect, it does seem to advance or speed up the rate of blastocyst formation in those mice.

Those are preliminary results so far but we certainly didn't see any detrimental effect unless we actually let the mitochondrial preparations sit for a while on the bench, and then what we think is happening is that you are starting to get leakage and cytochrome C which could actually be detrimental at that point. So, certainly from a cytoplasmic transfer point of view, I don't think you are going to damage the mitochondria at all because we are actually mechanically disrupting the cell membrane of these cells and centrifuging the contents to separate out

the mitochondria, and we don't seem to do any damage to the mitochondria in that situation.

Let me comment on the prior comment. I think you would have been surprised had there been homoplasmy; you would have expected heteroplasmy. In fact, we were the group that analyzed Dolly for heteroplasmy and we did not find it. It was homoplasmic. Those were sheep, and if you look at cows, they are heteroplasmic all over the place. So, it is the expectation to be heteroplasmic and it is something to worry about.

DR. SAUSVILLE: You referred to the experience with ICSI, which I interpret to be intracytoplasmic sperm implantation. Is that correct?

DR. COHEN: Injection.

DR. SAUSVILLE: Injection. Just from a sort of standard practice of this field, what would be the expected rate of major abnormalities resulting from ICSI as a process?

DR. COHEN: In the literature there is a range from 2 percent to nine percent. But a larger study, a study from the Belgium group who originated the procedure, with 3000 babies born, I think there was 3.4 percent, something around

there, and showed a significant increase in the rate of XOs.

DR. SAUSVILLE: But still that rate didn't go beyond a three percent sort of range?

DR. COHEN: No.

DR. SAUSVILLE: Thank you.

DR. COHEN: There is one publication that shows a rate of nine percent, but it was the same in the ICF population that was studied. That was a recent paper in The Journal of Medicine, in March. It was based on a small sample size but that is the only really high rate I know of.

DR. SALOMON: Dr. Moos?

DR. MOOS: First a comment on several of the remarks that have dealt with the characterization of the active principle. Cell biologists and biochemists have been fractionating very complex systems for well over a hundred years to see what part does what, and we are nowhere near the bottom of the pit. Nevertheless, even though we are shy of finding out where is the final proton and what it does, we have amassed a tremendous amount of very useful information.

So, I submit that a sensible way to look at it is to do the sorts of experiments that are

feasible and reasonable not just to enhance our understanding or to prove that this is good and that is bad, but to allow us to be able to develop some sense of what is necessary to keep consistent for a product to perform in a way that we can understand and predict.

A specific question that extends a point that was raised by Dr. Salomon and yourself, Dr. Cohen, since you brought up specific mRNA transcripts, has anyone evaluated whether injection simply of RNAs encoding some of the candidate genes you mentioned or pools of candidate genes has a beneficial effect on embryo quality?

DR. COHEN: Obviously none of those studies could be done in the human at this point. I am not sure that work like that was done. Certainly interference with mRNA was done, just the opposite, interfering with a specific RNA but I am not aware of injecting.

DR. SALOMON: Dr. Murray and then Dr. Van Blerkom and we will finish there.

DR. MURRAY: Dr. Sausville's questions about abnormalities associated with ICSI, I believe one of the studies, recently published, indicated the risk of low birth weight was also roughly

double, and that is after testing for multiple pregnancies.

The question I have for Dr. Cohen, I am asking for help in making sense of some of the numbers you presented about the incidence of heteroplasmy. You gave us a number--we don't have copies of your slides so this is from memory--something like evidence of heteroplasmy in 10-15 percent in a hypervariable region in the population. Am I recalling that correctly? The question is if you were to think about risk, obviously one of the ways one would think about risk is to say, you know, does this occur more or less often in the population that has been exposed to this particular intervention, ooplasm transfer, than the general population? I assume the hypervariable region is a non-coding region. Is that correct?

DR. COHEN: Yes.

DR. MURRAY: Therefore, you know, it may not be clinically significant. But here we have a heteroplasmy that is perhaps in a coding region, I assume if you are doing ooplasm transfer, so wouldn't we want also to have data that gave us some indication about heteroplasmy in coding

regions?

DR. COHEN: That has not been done, and that would be interesting. The work that has been done has all been on the hypervariable area.

DR. MURRAY: So, the 10-15 percent number doesn't tell us very much. It doesn't tell me very much.

DR. COHEN: No, but one thing that comes out is that it is an evolving field. Going by the literature, five, six, seven years ago the incidence was considered to be--well, there was no number but it was very rare to see this. So, now with new sensitive assays it is apparently much a higher frequency.

DR. MURRAY: But again, mutations in hypervariable non-coding regions are presumably not clinically significant, whereas what we would be interested in is evidence of mutations--

DR. COHEN: You shouldn't call it a mutation. It is hypervariable; it is not a mutation.

DR. MURRAY: Fair enough.

DR. SCHON: Can I just say something because I think I can clear this up? You transfer the whole molecule when you transfer mitochondrial

DNA, and you and I differ at 50 different bases in our mitochondrial DNA. Some of them happen to be in hypervariable region and some of them are in coding regions. So, you can't speak about mutations in mitochondrial DNA as being different. You get the whole molecule. If I transferred your mitochondrial DNA to me, I would get 50 different base substitutions on average. Some of them would be in the non-coding, some in the coding region.

So, the notion that 15 percent of babies that are born with heteroplasmy in a hypervariable region is just wrong. It is wrong. There is no evidence for it at all. The evidence is that somatic mutations, if you look at individuals and sample muscle or heart, for instance, you can find heteroplasmy in about 15 percent of those individuals perhaps at an extremely low level and it is in a single cell. It has nothing to do with the germline.

DR. SALOMON: So, it is not a safety issue.

DR. VAN BLERKOM: Just two questions. The donors were not mitochondrially typed. Right? These were random donors or did you type the mitochondrial DNA?

DR. COHEN: No, we didn't do that, no.

DR. VAN BLERKOM: So, this was after the fact?

DR. COHEN: Yes.

DR. VAN BLERKOM: Then the second question, maybe you can provide some basis or explanation as to why transferring a relatively small amount of cytoplasm would give you what you now see as 50 percent heteroplasmy, and do you think there is an upper limit on that? In other words, what is the upper limit?

DR. COHEN: The upper limit is 100 percent.

DR. VAN BLERKOM: So, as your techniques for sensitivity increase, is it possible that, in fact, it will be above 50 percent?

DR. COHEN: It is certainly possible, and it is certainly possible that there would be a drift over time.

DR. VAN BLERKOM: So, how could you replace this fairly sizeable replacement?

DR. COHEN: It is an enigma of the bottleneck, the mitochondrial bottleneck. That is where I think some of the clues lie. Replication doesn't take place until implantation of

mitochondria so the number of mitochondria that are suggested to be passed on is relatively small. I think Dr. Schon did some work on that, and I think it is a very small percent, less than 0.1 percent of the mitochondria in the oocyte that will actually make it to clonal expansion.

So, if you look at it that way, I think mathematically anything is possible. But it is certainly possible that there is a positive effect here. Everybody always likes to emphasize negative effects. Maybe there is a positive effect here and these are simply coming from a population that is more fit. That is a possibility. One thing I think Dr. Murray raised which is interesting is that we only found 3/13 and it looks very similar to maybe ratios that you would expect. So, it could just be a chance phenomenon as well.

DR. SALOMON: Thank you all very much. Even though we are off schedule, I don't think I would do it any differently, and that is just part of going into these very new areas where there are just a lot of really important issues that I think need to get set on the table early in order for us to do our job. So, I think this is fine. We will just have to deal with it a little later, and we

will. There is no free lunch in life, and certainly not on this committee.

But speaking of lunch, I am going to make an executive decision that we go to lunch now and then kind of put all the mitochondria stuff together after lunch. It is 12:50 essentially. If we can try and do this in half an hour and be back here--if you can just sort of eat and come back, we will start as soon as possible, as close to 1:20 as possible. Thank you.

[Whereupon, at 12:50 p.m., the proceedings were recessed, to resume at 1:40 p.m.]

A F T E R N O O N P R O C E E D I N G S

DR. SALOMON: If we can sit down again. Not that I am surprised, this is classic, we should have been back at 1:15 and here we are at 1:45. Anyway, I am sure there will be a couple of other people bopping in as we go along but we do need to get started.

The next speaker this afternoon is Dr. Eric Shoubridge, from the Montreal Neurological Institute, to talk about transmission and segregation of mitochondrial DNA. That will be followed by Dr. Van Blerkom, talking about mitochondrial function. So, we are going to kind of focus on mitochondria now.

Transmission and Segregation of mtDNA

DR. SHOUBRIDGE: I think my brief here is to tell you a little bit about what we understand about how mitochondrial DNA sequence variants get transmitted from generation to generation, and how they segregate in somatic cells and in the germline after that.

Most of what I am going to talk about is in the mouse model, a mouse model that we generated in my own lab, but I will try and relate it as much as I can to the human experience.

So, just so that we are all on the same page, a few people have mentioned the basic principles of mitochondriogenics but I just want to go over them very briefly. It is a 1000 copy genome in most cells. It is strictly maternally inherited. As has been mentioned, the male contribution gets into the zygote but it is destroyed by mechanisms that are still not well understood. The gametes are special cells, if you will, but the oocyte contains about 100,000 copies, at least 100,000 copies of mitochondrial DNA, and they are thought to be organized at about one copy per organelle, and the sperm contains about 100.

Germline and somatic mutations can produce mitochondrial DNA heteroplasmy. So, at birth, it is thought, that most individuals that are not carrying a disease mutation that they have inherited from their mom are homoplasmic. That is, every single mitochondrial DNA in the body has the same sequence. Nobody has really looked at this in great detail in thousands of individuals, but it is thought that most babies have in their bodies the same sequence in every cell, in every mitochondria. It is a highly polymorphic genome so each one of us at this table differs by about 50 base pairs on

average between our mitochondrial DNA sequences.

How does it segregate? It segregates for two reasons. One is that the replication of the genome is not very tightly linked to the cell cycle. In fact, it is not tightly linked to the cell cycle. What that means is that templates can either replicate or not during a cell cycle. So, what is controlled in a cell specific way is the total number of copies of mitochondrial DNA. So, neurons have different numbers than muscle cells, than fibroblasts and cells in the kidney, but they are turning over by mechanisms that we don't understand even in post-mitotic cells. The copy number is maintained but who replicates and who doesn't is not very well controlled or even understood.

So, in cells that are dividing there is an additional feature, that mitochondrial DNA is randomly partitioned at cytokinesis. So, we have two mechanisms that segregate sequence variants, both in cells that are mitotic and cells that are post-mitotic. That leads to this process that we are all interested in, called replicative segregation and the fact that the mitochondrial genotype you get at birth, if you happen to be

heteroplasmic, can be different in space and can change in time.

You already saw this picture that was produced in a review by Bill DeMaro, and we know now that mutations in mitochondrial DNA are important in a large variety of diseases. There aren't very many things we can say this, except that they can occur at any age and affect any tissue. That is sort of the worst case interpretation of this picture here but, in fact, these diseases generally affect the central nervous system, the heart and the skeleton muscle, tissues that rely heavily on ATP produced oxidatively.

The two questions I want to answer today are how is mitochondrial DNA transmitted between generations? The second one is what controls the segregation of mitochondrial DNA sequence variants in different tissues of the body?

It has been known for some time that mitochondrial DNA sequence variants segregate rapidly between generations. This was first established by Bill Houseworth and his colleagues in pedigrees of Holstein cows. What I want to show you, which is typical of the human situation, is a large pedigree that was published by Neils Larson,

from Sweden probably about ten years ago. It is a five generation pedigree that is segregating a particular mutation in tRNA. It is a point mutation that is associated with this phenotype called MERF and it has these clinical features.

There is a single person that is affected by this diseases in this five generation pedigree who has all of these features. What I want to point out here is if we just look at this line of the maternal lineage here, the numbers that are associated--and I am sorry, you can't see them from the back--the numbers that are beside these are measurements of heteroplasmy in the blood of these individuals. It turns out for this particular mutation, but it is not generally true, that what is in the blood correlates reasonably well with what is in affected tissues. It is always a little bit lower.

What I want to point out is this mom, here, who had a daughter with 73 percent of this mutation but a son with nothing. So, in a single generation there is nearly complete segregation of this mitochondrial sequence variant which happens to be pathogenic and produced a disease.

This mom, here, gave 73 percent to her mom

and then she had four boys, one of whom had quite a lot, 88 percent, enough to produce the disorder, and some who were asymptomatic even though they were carrying large proportions of the mutation that produces the disease phenotype. That is because of this so-called threshold phenomenon here. These guys were not affected because they didn't have enough mutant mitochondrial DNAs to produce a biochemical defect in the cells. So, another principle of mitochondrial genetics is that you have to exceed a threshold of mutants in a cell in order to produce a biochemical and, therefore a clinical, phenotype.

It turns out for the vast majority of mutations that we know about that that threshold is very high. So, if you have 70 percent or 80 percent of these mutants you can sometimes look completely normal, depending on how they are distributed.

In order to study this, a postdoc in my lab, Jack Jenuth, decided to make a mouse model. There are no known natural heteroplasmic variants in the inbred mouse population that we know about so we had to construct one. The way we constructed it was much along the same lines that we have been

talking about earlier today in humans. We found two different common inbred strains of mice, one which is called BALB and one which is called NZB, that happen to differ at about 100 base pairs, 100 nucleotides between the two genomes. We simply made, and we did this in both directions, a cytoblast from one of them. We injected that under the zona pelucida of the zygote here, and then we electrofused.

We don't know exactly how much cytoplasm we have put in here, but probably something on the order of 10-15 percent, which are the numbers which have been bandied around today. We fully expected to get transmission of this mitochondrial DNA that we put in here. In fact, we did.

So, we did this in a large number of animals. I just want to point out that these are the amino acid substitutions that are predicted by the sequence differences between these two strains. Here is NZB and here is another so-called old and inbred strain which is the same as BALB. They are, for the most part, at non-conserved sites in evolution, and for the most part conservative substitutions at those sites. So, in short, polymorphisms. The only one that is not is this

cystine for what is either an arginine or a leucine in this particular one, here. The rest look pretty much like polymorphisms.

So, we thought we were putting in neutral sequence variants. I must say, this is exactly kind of parallel to the situation in ooplasmic transferred humans. You are putting in a mitochondrial DNA that might differ at 50 or 100 positions in the whole genome. You are putting in the whole genome and this is very different than mutations that arise in the germline or somatic cells where you will get a single mutation on the same haplotype background. So, it is quite a different situation.

This is the first litter we got from one of our founders. We isolated several female founders. I can't remember exactly the range because it is a few years ago that we got, but this was pretty typical. We would get something like 3, 5 to 10 percent or so. Ten percent I think is the most we ever saw of the donor mitochondrial DNA in the founder females. We got that in most females. So, the expectation is if you put in, at least in the mouse model, 10-15 percent of cytoplasm you are going to get out something which is not so

dissimilar from that. It is a little bit less. Again, this is just a real eyeball estimate. We haven't measured anything in terms of how much cytoplasm we put in.

What we saw, and this is a very typical pedigree, is that some animals had completely lost that mitochondrial DNA and other animals in fact looked like they had amplified it. In fact, I will show you they don't amplify it, it is just a stochastic phenomenon. So, in one single generation, from a very small amount of mitochondrial DNA that is added to this, and this would be analogous to the human situation that we are talking about, you could in the next generation completely lose it or it can become more frequent in the offspring from that mom.

This pretty much parallels what we have seen in terms of transmission of pathogenic mutations in human pedigrees with disease. So, we wanted to sort out what the basis for this was, and the way we did it was using single-cell PCR. We simply went back in the female germline to find out what the level of heteroplasmy was in mature oocytes versus primary oocytes versus the primordial germ cells that were going to give rise

to the entire female germline.

The conventional wisdom was, as we knew from the observations, that there must be a bottleneck here somewhere because it looked like the 100,000 copies of mitochondrial DNA in the mature oocyte were not being transmitted to the next generation, if you will, because you couldn't possibly get rapid fixation for a mutation if the sample size of every generation was 100,000; 100,000 is a huge sample size. So, if ten percent of those were carrying a particular mutation and you sample the 100,000 in the next generation you are going to get about ten percent, plus or minus a little bit. So, it was pretty clear you must be sampling, effectively sampling a much smaller number and we wanted to determine what that number was.

I will just show you two pieces of data from that because it has been published years ago. Using single-cell PCR, we measured the proportion of heteroplasmy from the donor genome. In this case we have added the BALB genome on the NZB background. Here we are comparing what we see in the mature oocytes sampled from the female that produced these offspring. So, these are offspring

and oocytes from the same female mouse. You can see that the distributions pretty much overlap, meaning that by the time you are a mature oocyte there is no significant segregation of the sequence variant that we put in, that we donated to create the founder up to the point of the offspring being born.

We then went back a step further and we looked at primary and mature oocytes in the same animals by doing a little trick, and you can see here that the distributions also overlap. So, even by the time the primary oocytes are set aside, which happens in fetal life, all of the segregation of the sequence variants, of the heteroplasmy that is going to happen, that is going to be important in the babies that are born from this experiment, has happened. So, if you were to measure the heteroplasmy in the primary oocyte population, it would predict what it would look like in the offspring. Or, if you were to measure it in the mature oocytes, it would also predict what it would look like in the offspring.

I won't give you the rest of the data, but we went back and collected primordial germ cells and what we saw was that there was not that much

variation in the primordial germ cells, but by the time they reached this stage, the primary oocyte stage, all of the segregation has happened.

This is just a summary slide of what we think is the life cycle of mitochondrial DNA in the female germline. It is worth probably just spending a couple of minutes to work through it, just to refresh your memory about the things I have told you.

The mature oocyte, at least in the mouse, contains about 105 mitochondrial DNAs. The sperm brings in 100; they are completely destroyed. So, the zygote still has 105 mitochondrial DNAs and then, as has been mentioned before, there is no application of mitochondrial DNA in the early stages of embryogenesis. So, up to the blastocyst stage where the inter-cell mast cells are set aside, there is a reduction in copy number of mitochondrial DNA from about the 105 that is in the oocyte, here, to about 103, 1000 per cell which is, if you will, about the somatic number of mitochondrial DNAs in your average, if you can say there is an average, cell. But it reduces it from the very large number that is in the oocyte to here.

Then, when this implants we don't really know what happens, but we suspect mitochondrial DNA replication still doesn't restart and a small population of cells, called the primordial germ cells, are set aside. If you look at pictures of these cells in all mammals where it has been done, which is now in several species, they contain about 10 mitochondria. So, the mature oocyte had 100,000 copies of mitochondrial DNA and there are about 10 in these cells. So, this is where the bottleneck is. It is a natural physical bottleneck in the female germline. A very small number of mitochondria with a small number of mitochondrial DNAs, we think certainly less than 100 copies, are transmitted outcome the next generation.

These cells then start migrating from where they arise in the embryo to the general ridge, and they give rise to the complete germline population, called primary oocytes here, and at this stage, here, all of the segregation that is going to happen of the heteroplasmic sequence variants has happened. It is not going to be important further on. In mouse this might be 40,000 or 50,000 cells and six or seven million in humans. Most of those die by atresia and there has

been some thought that perhaps that cell death might be related to mitochondria, but I am going to argue in a minute that I don't think that that is important.

That is the state in the mouse. You can actually use some statistics to calculate the effective number of transmitting units between generations, but it depends on what model you use. So, that is just a statistic; it doesn't have any physical reality.

What happens in humans? Here are six common mutations, point mutations that occur in humans. Patrick Chittering and his colleagues in Newcastle analyzed the transmission of these mutations in all the published pedigrees, and I think this was published in the year 2000, all the pedigrees that they could find in the literature. They got rid of the proban so that they wouldn't introduce a big ascertainment bias, and if the transmission of the pathogenic mutations were the same as the neutral polymorphic mutations that we saw in the mouse, what you would expect is a symmetrical distribution around zero, which would be telling you that mom is just as likely to give more mutant mitochondrial DNAs to her children as

less.

In fact, that is more or less what you see here. It is a bit difficult to analyze this. This is not a random sample. These are people who show up in genetics clinics. The proban has been eliminated to get rid of some of that ascertainment bias but you can't completely get rid of it.

So, the point is that even though these mutations are pathogenic, it looks like the transmission of these mutations through the female germline is stochastic, just like it is for the neutral mutations that we studied in the mouse.

There is a single good example in the literature actually looking at the distribution of heteroplasmy in oocytes from a woman carrying a pathogenic mutation, and here is what you find. The mean proportion of this particular mutation of the mom in her oocytes was something around 14 percent, and you can see that a large proportion of her oocytes have completely lost it. Some had very little and some had more. I could take any of the mice that we looked at and plot the same thing here, and these distributions would absolutely overlap. In fact, if you used a statistic to calculate the effective number of segregating units

that could give rise to this distribution, it is indistinguishable in the mouse and human.

So, what we find in the mouse, as far as we know, looks pretty similar to what is in the human. So, the transmission of sequence variants between generations appears to be largely stochastic.

The effective number of mitochondrial DNAs in the germline is small because of the bottleneck that happens at the primordial germ cell stage. That causes rapid segregation of sequence variants. So, if an individual were to get, from whatever mechanism, mitochondrial DNAs from a donor individual, the next generation would now rapidly segregate those. So, some of her offspring may contain a lot of that particular sequence variant; some may contain none.

I think the evidence that pathogenic mutations are largely transmitted in a stochastic fashion, which is almost indistinguishable from what we see in the mouse, suggests that there is no strong selection for mitochondrial function during this process. So, what we are talking about here, one of the aspects of what we are talking about here today is whether the additional boost, if you

will, that could be given to a zygote from a small amount of extra mitochondria there, I don't think in the disease cases there is any reason to suspect that that is true because there are lots of babies born who are perfectly normal, who later get into trouble, and they might get into trouble even in the first few months of life but they may be carrying 90 percent or 95 percent of mutant mitochondrial DNAs. If those mutants are organized as one per mitochondrion, then certainly those mitochondria have very little function. So, I think the point is you don't need much mitochondrial function either to go through oogenesis or to go through fetal life and have a perfectly normal baby. Later on things can happen, and they do in disease.

What about segregation? What about after, in post natal life? Well, if you look at human disease, there are any number of patterns of segregation of pathogenic mutations. Let just focus on two that I have on this slide, two common mutations in tRNAs that are associated with well-recognized clinical phenotypes. One is the point mutation in lysine that is segregating in a pedigree that I showed you earlier on. Here, it

looks like the affected individuals have high proportions of this mutation in their skeletal muscle, always over 85 percent. There is also a high proportion in the blood which is usually about ten percent less than what is in the muscle.

If you contrast that with another mutation that is again a point mutation in the tRNA that produces a completely different clinical phenotype, it is all over the map in the blood, the proportion of these mutations. There is a high proportion in rapidly dividing epithelial cells and they can collect in the urine. We don't know what that looks like for this particular mutation, and it decreases with age in the blood, whereas here we don't really have any evidence that there is much of a change in the proportion of these mutants with life.

So, here are two different tRNA point mutations. They have been worked on quite a lot. We know that they produce translation defects in mitochondria and, yet, the segregation of these sequence variants, the pattern of segregation is very different. You wouldn't really predict that if the segregation pattern depended upon function, mitochondrial dysfunction, if you will, in some

way. The pattern of segregation we know determines, in muscle at least because this is the tissue we have the most access to, what the muscle phenotype looks like.

Here are muscle biopsies from two patients that are carrying this tRNA lysine mutation that is associated with MERF. One of them has this very typical pathology called ragged red fibers. These are grossly abnormal muscle fibers. If you stain them for cytochrome oxidase activity, which is one of the enzymes in the mitochondrial respiratory chain, they are completely negative. They have absolutely no activity. And, you have huge proportions of these mutants here.

There was another patient who had completely normal muscle biopsy, but the proportion, if you just took a piece of muscle of the mutation in both biopsies they are virtually identical. So, how they distribute in muscle and presumably other tissues determines, to a large extent, what the phenotype or how serious the biochemical phenotype is and presumably that determines some of the clinical picture.

If we look again, comparing these same two mutations with age, and Here Joanne Pulsion, in

Oxford, first did this plot and she said, well, if there is no real pattern to what is going on in the blood of patients carrying this particular 3243 mutation, maybe what is happening is that it is changing in the blood and it is changing in the muscle as well. So, the difference between what you find in the muscle and the blood might be linear with age. In fact, that is, indeed, what she saw. Subsequent studies have shown that this mutation does decrease in age with the blood and probably increases with age in the muscle. The mutation they talked about at 8344 doesn't do anything with time. It is absolutely flat. So, the evidence there is that what you get at birth determines how sick you will be, whereas things can change with other mutations.

So, that is all extremely confusing. You are probably confused so here is the conclusion: there is no simple relationship between the oxidative phosphorylation dysfunction and the pattern of segregation. There are lots of different patterns and we don't understand what it is. It could be some subtleties associated with the mitochondrial dysfunction that mutations produce, or it could be that some other nuclear

genes are controlling this whole process, and that is what I want to talk about in the last little bit.

To come back to our mouse model of segregation, when Jack Jenuth was in the lab and we had sorted out the transmission we thought, well, that is kind of neat. We expected to see something that was different and stochastic and we didn't and we thought let's look in the tissues and see what we see. We expect it would just be random there, just like it was in the female germline; there wouldn't be any particular pattern and sometimes the proportion of this sequence variant would go up and sometimes it would go down, and whatever tissue we measured, it would be all over the map.

In fact, we saw something completely different. If we looked at rapidly turning over cells, like colonic crypts, we found out that that was the segregation which turned over in the mouse about once every 24 hours. That was completely random. If we then looked later on at age, what we found was that most of those crypts had completely lost the mutation but a few were going towards fixation of the mutation.

So, this is a picture like you could see

in a population in a textbook, and this is the probability of fixation of a rare neutral mutation in a randomly mating population, and this shows it par excellence. So, the proportion of crypts that should be fixing the mutation should be directly proportional to the initial frequency of the genotype in that population, which was about six percent and that is about what we saw here. About six or eight percent, I can't remember the exact number were fixing but most of them had lost it by pure random genetic drift so there was no selection at all involved here.

If we looked in the brain, the heart and skeletal muscle we couldn't even see any evidence for that in the lifetime of the animal. Very surprisingly, if we looked at a few other tissues like the liver, the kidney, the spleen and the peripheral blood we saw a very strange phenomenon that had never been described before, and that was tissue-specific and age-dependent selection for different polymorphic mitochondrial DNA genotypes. So, the liver and the kidney without exception would select for the NZB mitochondrial DNA and the BALB in the spleen, without exception would select for the BALB, the opposite mitochondria in the same

animal. So, we didn't know what that meant.

Then a graduate student came to my lab, Brendan Battersby, and picked up on this and he wondered what was going on, what was selecting for this particular sequence variants that we would have predicted would not have functional consequences, and that were transmitted through the female germline as completely neutral variants in a completely stochastic fashion.

So, he did some sing-cell PCR in the liver and basically wanted to measure what the increased fitness was for the NZB mitochondrial DNA which, as I mentioned, always selects in the liver. By 18 months, most of the hepatocytes in the liver are fixed for that, and it doesn't matter where they started--they could start at one percent or two percent, if you look a year and a half later, they are fixed. So, it happens at a constant rate. It is independent of genotype frequency, which is very mysterious if it were a function but it is not what you would predict because of these threshold phenomena.

Initially we said, well, that can't be related to function. So, we measured this relative fitness simply by comparing the initial and final

genotype frequencies in animals. The way we got the initial frequency was to look at tissues where these things weren't segregating. So, we assumed that is what the animals were born with. By the way, we have pretty good evidence--we have data actually to show that at birth all tissues are pretty much the same in terms of the level of heteroplasmy of these patients. So, if you get two percent or five percent or ten percent, every tissue has the same amount and you would predict that amniocytes would have the same amount too. There is a little bit of data in humans to suggest that that is true.

So, if we measured this relative fitness for this thing at two, four and nine months of age we pretty much got the same answer. So, there is a constant advantage for this genotype in the liver. Every time mitochondrial DNA turns over this particular genotype has a 14 percent advantage. So, if you wait long enough, no matter where you start, it will fix for that mitochondrial DNA genotype.

If you look at oxygen consumption, a fairly crude way to look at function of mitochondria, this measures Vmax of the respiratory

chain and we couldn't see any difference. So, we put essentially a very high proportion--we couldn't get 100 percent at the time we did these experiments, NZB mitochondrial DNA on a BALB nuclear background or 100 percent BALB on a BALB nuclear background, and these are just measures of mitochondrial respiratory chain function, and there was no difference, which is what we also would have predicted.

We then thought maybe it is just replication, although the base substitutions in these two different molecules did not affect any of the known regulatory sites that have been defined in the literature, but we thought maybe we will measure replication and see if there is a difference anyway. So, we did an in vivo experiment where we injected with BrdU to label mitochondrial DNA. We isolated mitochondrial DNA and did a Southwestern analysis. So, the idea here is that we are looking at incorporation of BrdU in the mitochondrial DNA. We strip this and then we just do a straight Southern to look at how much mitochondrial DNA is there.

We have two different sequence variants that we can recognize because there are restriction

fragments here. So, we have the NZB or the BALB mitochondrial DNA. These are five different animals obviously because we had to sacrifice them at different times during the experiment, but the number to compare is this versus this. So, if there is no replicative advantage in this experiment for this molecule, for the NZB molecule over the BALB, then the incorporation rate of BrdU should reflect the proportion of the genome that is there and that is, in fact, the case. So, we have no evidence that this is based on replication.

If you take hepatocytes out of these animals and culture them you get exactly the opposite effect. They now select for the BALB mitochondrial DNA and not the NZB mitochondrial DNA--completely unexpected. We don't know why this happens. It turns out it is not so easy to grow mouse hepatocytes so I am not going to pursue that, but you can actually calculate the relative fitness. The copy number of mitochondrial DNA drops when hepatocytes start to proliferate in culture and the relative fitness goes up by about a factor of two, but for the opposite mitochondrial DNA.

So, this was all very mysterious. We got

a small advantage for the this NZB thing, not based on function as near as we can tell. It is not based strictly on replication. If we change the mode of growth in the genotype the selection can be opposite. So, what we concluded from all of this was that selection must be acting at the level of the genome itself. It doesn't have anything to do with the function. It is not acting at the level of the cell or the organelle; it is acting at the level of the genome.

So, we hadn't made any progress with the biochemistry here so we needed to do something else, and my son summed this up very well. He was working with his mom in the kitchen one day. He was making a cake and he turned around and he said, "Daddy, you know, every experiment has a wet part and a dry part."

Here is the dry part, the genetics. We had to turn to genetics. So, the idea now was to see if we could tease out a gene, a quantitative trait locus that would determine whether or not you would select for the BALB or the NZB mitochondrial DNA in a tissue-specific way.

So, here is the breeding strategy but it is a pretty typical thing you do in genetics. In

mouse genetics you just breed two inbred strains together and look in the F2 generation and see if the phenotype, and the phenotype here is tissue-specific directional selection of mitochondrial DNA, see if it segregates. In fact, it does.

I will just show you the example in the liver in the interest of time. This is a random collection of about 50 animals. Actually it is a little bit less, they are not all in the liver here; from muscle in animals at 3 months or 12 months of age. These are F2 mice in this experiment. The muscle is not doing anything interesting but you can see that at 3 months there is kind of a bimodal distribution in the liver, and by 12 months they are completely fixed to that genotype. I won't show the other tissues in the interest of time.

We then calculated the relative fitness using the same measure that we used before in these animals and it looked to us like in the F2 animals there were some that looked like the parents that were selecting, strong selectors for the NZB genotype; there were others that were weak selectors for the NZB genotype; and then there were

some in the middle. This kind of looked like a one to one distribution to us, which is suspiciously Mendelian, and we thought maybe there is a single strong gene that underlies this effect, a nuclear gene which is controlling segregation behavior.

The idea would be that in the BALB animals are behaving like a parent, and the animals that we bred them with, which are actually a subspecies called moos-moos castenius, were homozygous for the castenius. So, we tested that. We did genome scans that were done on all the tissues. I am just going to summarize the data rather than going through how we did this genetically because I don't think anybody is particularly interested in those details and if you are, you can ask me.

We did a genome scan at three months in the liver, and what we found was a locus on mouse chromosome 5, which a giant LOD score which, those of you who know about LOD scores, that is pretty big; I haven't seen too many that are bigger, that explained almost 40 percent of the variants of that genotype. In the kidney we saw another locus on chromosome 2 that explained less. This acted in a dominant way; this one was recessive. At this stage we couldn't really score the phenotype in the

spleen accurately so we didn't really pick up any linkage.

If we look at 12 months of age we don't see any linkage in the liver because all the animals are fixed so the segregation has all happened. That is telling us that the BALB allele is presumably a strong allele than the castenius allele but eventually the castenius alleles catch up. But we saw the same locus in chromosome 6 that could account for 15-20 percent of the variants in the kidney and the spleen and it was the same one, the same locus. If you remember, these are going in opposite directions. So, this is selecting the NZB mitochondrial DNA; this is selecting the BALB mitochondrial DNA.

We ended up with three quantitative trait loci that explain a fair proportion, especially in the liver, of this variation that seem to control the selection of what we think are neutral variants of mitochondrial DNA. How that happens is a complete mystery so far. We don't know what they look like. They are probably not molecules that are involved in the replication of it because it looks like the replication is the same. So, we think they may be codes for molecules that are

involved in its maintenance.

So, this is a summary of the three different loci on three different chromosomes. One of them, in the liver, is very highly significant; the other ones are significant by the normal criteria used in quantitative trait analysis.

I will just conclude, just to sum this whole thing up, the transmission of these sequence variants in the germline, as I said, looks like it is completely stochastic to us. There doesn't seem to be any bias one way or another. We have now actually bred animals completely in the other direction. So, we have put in a couple of percent of the NZB or the BALB--now we are just doing it with NZB on a BALB background into founder females and we can get 100 percent if we just breed for a few generations of NZB on a BALB background starting out with two. So, it doesn't matter where you start from. Because it rapidly segregates through the germline in a stochastic way, you can just pick animals that have a high percentage and the offspring from those mothers are going to have a higher percentage, and in about three or four generations you can get 100 percent the other way.

There is tissue-specific nuclear genetic

control of this segregation process which does not seem to be based strictly on replication of the molecules or on function of the molecules, which is very surprising, and we think, but this is not just hand waving, that perhaps the genes that code for these molecules might be involved in the organization of mitochondrial DNA in the nucleoid. There could be a lot of other things and we are now trying to clone those.

In closing, I just want to acknowledge the two people in my lab who have done most of this work, two very talented students, a graduate student, Brendan Battersby and a postdoc who started it, Jack Jenuth. Thanks very much.

DR. SALOMON: I never know whether to clap or not, but as we didn't clap before--

[Laughter]

--that was a very nice talk. Just in terms of trying to be efficient with time, given that the next talk is also about mitochondria and you are sitting on the panel with us, unless someone has a question which just totally be out of context and they just have to ask it now--I don't see anyone jumping up because of the way I put that, I guess--I would like to go on and have Dr.

Van Blerkom give his talk and then what I think we need to do is stop and talk a little bit about what does this tell us now about mitochondria and how this specifically relates back to safety and other issues with respect to ooplasm transfer.

Mitochondrial Function and Inheritance Patterns in Early Human Embryos

DR. VAN BLERKOM: Thank you very much. Let's see if I can put a number of different aspects of human development and mitochondrial function, other than necessarily respiratory function, in the context of what this is all about, which has to do with cytoplasmic transfer. So, I would like to talk a little bit about the oocyte, since we are really dealing with the human, and the types of information that we can gather from available studies on the behavior of oocytes and their biology.

In the human this is what we deal with initially in the IVF lab, which is the mast cells, the cells surrounding cell structure, about 100 microns in diameter, which is the oocyte. You can see it right here. What we know now from a fairly substantial amount of biochemical and physiological studies is that, in large measure, the potential of

this oocyte that is here, its developmental competence is largely determined by factors that have occurred before this egg has even been ovulated. So, influences to which this oocyte is exposed in the follicle during oogenesis actually largely determine its competency after fertilization. We know this from studies of biochemistry on follicles, the physiology of blood flow, some of which have been used as predictors of oocyte competence in trying to select oocytes such as these from many that may be retrieved from an IVF procedure.

The next slide shows a picture of an oocyte, and here is the problem. I mean, when you look at this egg here, this human oocyte it is normal in appearance. It has a polar body and everything else that it should have. Most eggs look equivalent but their potential is different. We know that some of these eggs will be competent to go on and divide normally and implant. Others that look the same don't. This is the notion of why you might want to rescue the cytoplasm, or there may be a cytoplasmic defect of some sort in these eggs that render them incompetent.

One of the things that Jacques Cohen