importance to that? Or are these bone marrow stem cells, which we know are circulating, of course, feeding these stem cell populations in the different organs in the adults? I hope that we'll come back to that. That's certainly an area that I'm interested in.

So, about a 10-minute break and then we'll start again.

(Recess.)

DR. SALOMON: Welcome back to the second part this morning.

I'd like to introduce the next speaker which is Dr. Mehandra Rao to talk about the characterization of lineage-restricted, self-renewing precursors from neuroepithelial stem cells.

DR. RAO: The title might suggest that all I'm going to talk about is the work that we've done. I just took advantage of this opportunity to try and sort of make a few specific points about cells present in the nervous system and see whether these points are relevant to how they might be applied for therapy. So, I'm not going to talk a whole lot about actual data, but most are general points, hopefully what people will agree is a consensus in terms of stem cells.

This is sort of stem cell 101. If you go through normal differentiation or you look at development

of the nervous system, you can start with what Dr. Gearhart already told you about, cells in the inner cell mass which will give rise to all cells in the adult. These cells can be either grown in culture as ES cells or you have related cells like the primordial germ cells, which can then give rise to all the differentiated cells in the nervous system, as well as in other tissue.

These cells don't do it directly, though, at least in the normal embryo. They differentiate and there are stages of differentiation that have been identified. You initially have differentiation with three basic germ layers. You have ectoderm, endoderm, and mesoderm. It's the ectoderm which generally gives rise to the future central and peripheral nervous system.

You get differentiation into the epidermis, and then you have three different components of the nervous system. You have the central nervous system itself, and you go through several stages of differentiation before you get differentiated neurons, astrocytes, and oligodendrocytes.

The peripheral nervous system is derived from two different populations of cells. It's the neural crest cell which gives rise to PNS neurons and glia. In addition -- and this is an important to remember -- we also get several non-neural derivatives, which include smooth

muscle, cartilage, and bone, which are derived from neural crest. Placodal cells give rise to the cranial ganglia and in addition give rise to non-neural derivatives.

So, you can see that you can get neurons from a wide variety of cells which are there at different stages in development. They're all functionally similar in the ability to give rise to neurons and glia, but they're clearly different and they have different roles in normal development.

In addition to the sort of normal process of development, you also have, as you heard already, a process which seems to be more common than we had originally thought and that's the process of transdifferentiation or cells which would normally give rise to other tissue giving rise to nervous system derivatives, or vice versus.

So, we have neural stem cells, which can maybe perhaps transdifferentiate to give rise to mesodermal derivatives, a thing they normally wouldn't do in development. We don't know how exactly that would happen. It could be either the process of transdifferentiation or it could be dedifferentiation back to a more primitive cell which then subsequently undergoes differentiation.

One thing we need to keep in mind, at least in terms of stem cells differentiating in this direction, is it could be a normal process of differentiation too. So,

you could have CNS stem cells give rise to PNS stem cells like crest, which could then give rise to mesodermal derivatives.

I want to follow the development for a couple more steps just to emphasize a few points. As soon as you get ectoderm differentiating into epidermis and neuroectoderm, that's normally present as a sort of plate of cells which subsequently undergo a process of morphogenesis to form a closed neural tube. At this stage, cells seem pretty primitive. And people have characterized the properties of these cells, and at least in normal development, it looks like these are functionally neural stem cells. They can give rise to multiple derivatives. They can self-renew, and they can give rise to both the CNS derivatives, as well as peripheral nervous system derivatives.

Development proceeds further in the nervous system so that what you had initially as a homogeneous tube then undergoes subsequent sort of flattening events, and you get differentiation and more differentiated cells which arise. It occurs in a specific pattern, and I don't want to go into any details except to say that there are specific regions of the neural tube which will give rise to subpopulations of cells which will then undergo further sequential processes of differentiation to give rise to the

more mature phenotypes present in the nervous system.

We and many other groups have been able to isolate cells at different stages of this developmental process. So, you can, for instance, identify neural stem cells, and we can define them as cells which can give rise to all the major derivatives which are present in the nervous system.

You can also go in at slightly later stages of development and identify more differentiated cells. So, for example, you can identify lineage-restricted cells. In this case, this is a neuron-restricted precursor cell which can give rise to predominantly neurons.

I again use the term "restricted" only as a soft term. It's not an absolute term. It's maybe lineage-biased and we define that bias as a comparative bias. If you take two populations of cells, both of which can generate neurons, and if you challenge them to a certain condition, if one is biased towards giving predominantly neurons while another cell gives rise in the same environment to other kinds of cells, we say it's biased towards one fate or the other.

Likewise, you can identify glial precursor cells. These are cells, which in the same environment in which neuron precursors would give rise to neurons, will readily give rise to glial cells. We define glial cells as

astrocytes and oligodendrocytes, which are two separate populations of cells.

Overall, when we've looked at all of this development, there's just one important point I want to make from this slide. It's that there is a lineage relationship between these kinds of cells during development. The most primitive cells we've called neuroepithelial cells are present very early in development. They can give rise to all the differentiated cells that you see on the right here, and they undergo a sequential process. We and others have shown that you can generate all of these sort of more intermediate precursors from this early precursor cell and that these then are relatively more restricted in their differentiation potential compared to this cell. Nevertheless, they still have the ability to differentiate into more than one phenotype.

We've shown the lineage relationship between these sort of particular cells, but I don't want to leave you with the impression that these are the only lineage-restricted precursors that exist. There clearly are likely to be several other classes of lineage precursors.

I want to make one more point here and I'm going to use two slides to do this. Though all of these cells can make neurons, we can identify functional

differences or antigenic differences between these cells. So, they're present in different parts of the body. They're present at different stages in development, and we have antigenic characteristics which will distinguish between these cells.

Here's just an example of differentiation between a neuroepithelial stem cell and a neuron-restricted precursor cell in terms of three different markers that you can look at. So, both of these cells are similar in that they express nestin. This cell, however, expresses additional neuronal markers which the early precursor cell does not express. There are differences between the factor responses to these cells in culture and in terms of their functional ability. And this is going to be important later in the talk.

If I can summarize here then, there are multiple cell types that can generate functional neurons and glia. These include embryonic stem cells, multipotent neural stem cells, more restricted precursors, and also these two things which often get forgotten, crest cells and placodal cells.

Placodal cells are quite important because there's been a lot of excitement in the field with olfactory ensheathing cells. I want to remind people that olfactory ensheathing cells actually derive from a placodal

cell.

In addition, we have cells which may not normally give rise to neurons and glia but can do so by a process of transdifferentiation. These include -- you've already heard about mesenchymal stem cells, mesodermal stem cells. I've added others here just because people have also used cell lines as a source of neurons and glia.

I want to add that this already seems to make life somewhat complicated, and I'm going to say that life is a little bit more complicated even than this. That's that we don't just have stem cells and restricted precursor cells, but as people have talked about just in the last couple of hours, the environment and the time at which you isolate these cells, the most subtle properties of these cells may be different.

One example of that is clearly true, for example, with multipotent cells. We identified multipotent cells as being FGF-dependent cells which are present at a very early stage in development. Several other groups actually identified a stem cell which is present a little bit later in development which is present all the way through the adult. It is not FGF-dependent, but it's EGF-dependent. Clearly, we know that these are different cells, and several groups have shown that these are different cells. So, not only do you have multipotent

cells, but you have classes of multipotent cells.

This is just a summary of the differences between at least two classes of multipotent cells.

The next slide just gives you a listing of the various kinds of multipotent cells that have been defined. So, we have FGF responsive cells. We have EGF responsive stem cells. We have maybe an E-NCAM immunoreactive cell. That's how people have defined it, which is present in the adult cortex. It was defined by a group at Albert Einstein. There are cells which are present in the SVZa in the adult, which may be somewhat different. Human CNS stem cells seem to be different from rat and mouse in their growth characteristics and the growth factor requirements. And several other cells.

All of them in many ways are functionally similar. They can give rise to neurons. They can give rise to neural crest derivatives, astrocytes, and oligodendrocytes, but clearly we can identify differences in self-renewal capability and growth factor responses.

The other somewhat disturbing thing for us was that this was also true for more restrictive precursor cells. So, we could go a little bit later and look at neuron-restricted precursors and ask is one neuron precursor cell virtually identical to any other neuron-restricted precursor cell. The emerging evidence, at least

in our minds, is it's not absolutely true. The properties of a neuron-restricted precursor in the sense of the kinds of neurons it makes or the synaptic connections that it can make are quite different, and these differences depend in terms of where these cells were isolated from.

We try and define neurons, and that I guess is a point that came up in the talk here, how do you define a neuron? You can't define a neuron basically just from the expression of markers, but you really need to look at several properties overall. In our minds, for neurons at least, you should be looking at electrical activity, the ability to generate action potentials, the ability to receive synapses and the ability to make synapses, and the ability to synthesize and release neurotransmitters. We normally try and use these criteria to say that this cell can generate neurons and what kind of neurons it can make.

This is just an example of getting action potentials from a neuron-restricted precursor cell.

So, when we look at cells and the neurons that they generate, we find you have to look at several different properties. You have to look at whether they can project axons and whether they can make synapses and use markers to do that.

We also use criteria in terms of adding neurotransmitters and looking at the response profile using

sort of fura imaging, and this just shows you examples of cells responding to different neurotransmitters.

The importance of this is not just to say that we can do it, but it's to make one additional point. Even in culture, where we think we have a relatively large amount of control and in some sense they're all in the same environment, if you actually examine the neurons that are formed in a dish, you can look at two adjacent cells here, and their neurotransmitter response is different. So, clearly there is heterogeneity despite what we think of as a reasonable amount of control in our ability to differentiate cells. Since neurons are defined by the properties that they have and their ability to make synapses and the kind of neurotransmitter they synthesize, it means we don't know how to regulate this process of differentiation well enough that we can guarantee we have 100 percent of a particular class of neurons.

This slide just summarizes where different neuron-restricted precursors have been isolated from, different groups of isolated precursors from different parts of the brain. The importance here is that these differences are actually biologically and functionally relevant, and I'll come to that in a little bit in the next few slides basically.

The same thing is true for glial restricted

precursors, and Dr. Noble made this point earlier where he said there are several types of glial precursors. And this just summarizes the results from several different labs. For simplicity's sake -- again, it's by no means complete -- I've classified glial precursors as sort of maybe three kinds. There is a glial precursor which can give rise to both oligodendrocytes and astrocytes, another precursor which seems to give rise to predominantly oligodendrocytes, a third precursor which seems to give rise only to astrocytes. I want to remind people that there are other glial precursors which may be therapeutically important, and that includes a Schwann cell precursor cell or olfactory ensheathing cell precursor.

Again, there's a huge body of evidence that these cells are different from each other. We initially looked at these differences in culture and clearly they were there. But I think that the other important point I'd like to make here is that the response to the same environment can be different, so there are intrinsic biases in cells, and this bias is not just a reflection of a culture artifact, but it's actually true when you transplant cells back in the host environment.

A clear-cut example here is simply this, that if you take labeled cells, which have been labeled with GFP, and these are neuron-restricted precursor cells, and

you put them back in the brain, if you put them back in one particular environment, they'll only make neurons. On the other hand, if you take a glial precursor and put it back in the same environment, it will actually make astrocytes. And you can see that double-labeling here.

So, what this tells us is that when we defined these cells as neuron-restricted precursors or glial-restricted precursors, that this is sort of a functionally important classification because, in the same host environment, their response to that environment is going to be different.

As I pointed out earlier, there are these several classes of neuron-restricted precursors or glial-restricted precursors. We've also tried to compare whether the behavior of these cells would be the same or different, and these were experiments we did in collaboration with Dr. Marla Luskin. We just simply asked if we take spinal cord neuron-restricted precursors and put them back in the subventricular zone, will they behave like the subventricular zone neuron-restricted precursors or will they behave differently. The answer was that they behaved differently.

Normally if you have subventricular zone precursor cells and either the endogenous precursor cells or ectopically transplanted precursor cells will only

migrate down this sort of stream toward the olfactory bulb, the rostral migratory stream.

On the other hand, if you take spinal cord precursor cells and put them back in that same location, they will migrate up to the cerebral cortex. They'll migrate to several different regions. This migration is not random. It's specified. So, clearly the environment is directing these cells, but the way the cells read the environment depends on where they were isolated from and what their intrinsic properties are.

The low part is just to show you controls to show that we labeled them with GFP, that they look like neurons. We look at staining to see what kind of neurons they made, et cetera.

So, if I can summarize this part -- I'm going to cite a couple of other people who have done similar things here -- is that cells show an intrinsic bias in their development even when they are exposed to the same environment. This bias is seen very early in development. If you take precursors which have been isolated from the embryo, you can see this difference or bias in differentiation if you take stem cells which have been isolated from different parts of the brain. It looks like, at least to us, that the bias is retained in culture for several passages, at least for 3 months in culture. As far

as I know, we don't know how to reverse this bias. I think that it's really important for us to understand this bias because this will enable us to predict the behavior of these cells after transplantation.

The next point -- and this again became an important point, in fact, in conversation earlier here -- was that not only do cells have an intrinsic bias in their differentiation potential, but the expression of the potential depends on the environment in which the cells are put. The brain cannot be treated as a homogeneous region and say, well, one part of the brain is the same as any other. There's clear-cut evidence that the cells will behave differently depending on which microenvironment, so to speak, in the brain you put them back in. I'm just going to give you a couple of examples where I think this is very true.

It looks like stem cells, when they're put in the ventricular zone, behave differently than if they're put into the cortex. The rate of cell division is different. The long-term integration is different. I'm not going to show you a whole lot of data, but there's a variety of data which says that that's true.

A another very quite important point is this thing about neurogenic versus non-neurogenic regions and injured versus uninjured, which was again a point that came

up here. So, if you take neuron-restricted precursor cells and put them in different parts of the brain, the kind of neurons you'll see will be different. So, for example, when we put them in the cortex, we see predominantly projection neurons. However, if you put it back in the cerebellum, you see predominantly glutaminergic cells of a particular kind. Again, I'm going to skip over all the staining and the markers we used, but clearly we know that this can happen.

The other point, as I said, is this neurogenic versus non-neurogenic region. I showed you all this data where we took neuron-restricted precursors and put them in the brain, and they made multiple kinds of neurons. However, if we take the same cells and we put them in the spinal cord, a region where you don't see a whole lot of ongoing neurogenesis, we don't see neurogenic differentiation. So, the same cell responds to the environment differently depending on which environment it has been put into. I think several other groups have similar data.

The same point we can make with glial precursors. Depending on the response of the cell to the environment, glial precursors might make astrocytes or they may make oligodendrocytes. So, the reflection of the potential of the cell depends on some kind of environmental

signal that it receives.

So, keep this in mind then. We have many, many classes of cells. The classes of cells that we have are far more than we thought. Those cells have an intrinsic bias, which is reflected in the differentiation potential, and the environment can modulate this differential bias. So, we have this huge population of different kinds of cells that we have to worry about in some sense.

I want to also say there's one additional complication we need to keep in mind. We have many different therapeutic targets that we want to use cells for, at least in principle. That's just a listing and it's just taken from Harrison's Textbook of Internal Medicine, which talks about different neurological disorders. Different groups have argued at different times on whether we can use stem cells or their derivatives as therapy.

I want to say that if you take even one of these single therapeutic targets and ask what do we want these cells to do, we find that there's quite a large number of things that we expect a cell to do. Because of my interest in spinal cord, I have chosen spinal cord injury as a target and said we'd really like ideally to say we could use stem cells as therapy. We really want to reduce the scar formation. We want to maintain synapsis, reduce axonal degeneration. We want to have remyelination

of the demyelinated axons. If there's a cavity, we want to be able to pack that cavity. Sometimes we want to give growth factors and reagents, and maybe we want to use cells as a delivery agent to introduce foreign genes or we want to mobilize the endogenous stem cell population maybe because of the cytokines that these cells secrete.

I want to make a statement, and maybe I'm going to get grief for this. We'll see I guess.

(Laughter.)

DR. RAO: The strong statement I want to make is that there won't be a single cell for therapy. It's just not going to work. There's no one size fits all for any therapy that you want to use in the nervous system.

The next statement I'm going to try and make is that the choice of cell will depend on the cell therapy or the goal that you have. For whatever is the therapeutic outcome that you want, you will have to choose the right kind of cell for that therapy.

For all transplant therapy, there might be some general rules which we'll have to worry about in terms of doing this, and that's what will be the cell behavior after transplant, the degree of cell death, its rate of proliferation, its ability to migrate, its ability to differentiate appropriately and the lack of inappropriate differentiation. This is really important to keep in mind.

You've heard of different sources of cells, mesenchymal cells, transdifferentiated cells, neural stem cells, and how would they respond to the environment. We always look at the positive and say, well, they did make neurons, they did make astrocytes, but we also have to ask what else did they make? What is that large group of cells which are differentiated? What are they going to do?

There are two other issues which normally don't become major issues when we think about transplants but I think are important in the nervous system, and that's a method of insertion. It's really quite clear that we have a limited strategy in how we can insert cells or deliver cells, and the method of insertion can be quite critical in terms of survival, how you dissociate the cells, in terms of how you treat the cells beforehand. I think we need to really worry about that as an issue in combination when we're looking at anything in terms of a clinical trial or looking at animal models.

These two things which haven't come up as questions were the immune response that these cells will generate. And it will become clear why I think that's an important issue. But I've gone through the literature -- and maybe I'll be enlightened today, but it's very hard to predict the immune response of early cells which are primitive or undifferentiated. There isn't a sort of

standard cell assay that we can do for immune response for a graft versus host or host versus transplant cells, which is a ready, easy assay which we can use. I think that we have to worry about this and we have to consider what's going to happen when we do long-term transplants with both major and minor histocomparability antigens.

There's also clearly a growing body of evidence which suggests that a lot of genes which drive expression are shut down. So, if you use these cells for gene delivery, you have to worry about that as an issue too.

In addition to these sort of more general properties of cells that we'll have to worry about in any case, I think specialized cell populations will require sort of specialized tests that we'll have to worry about. I'm going to just give two examples.

One is embryonic stem cells. Dr. Gearhart already raised this as an issue. What will happen? Will you get inappropriate phenotypes that may develop? Will you see tumor formation, and should we be designing specific tests which are relevant to the known properties of specialized populations of cells? I think in the case of ES cells it's very clear that you have to worry about tumor formation, and that will be an assay that you'll have to run.

With the issue of transdifferentiation too,

we'll have to worry about a couple of things. I just want to make the point about numbers and cytokines. The issue of numbers is that we clearly know -- at least the data is quite compelling -- that you can get transdifferentiation and you can theoretically use, for example, mesenchymal stem cells to give you neural derivatives. But what sort of gets lost is what is the percentage. What is the degree of efficiency? Are we going to get all the cells becoming the kind of cell that you want? Are the numbers going to be something which are useful?

The other for me conceptually that is a major worry is that when you go back and look at the cytokines that regulate differentiation -- you heard today EGF and FGF seem to work to get you bone marrow formation and you get stem cells proliferating which can give rise to mesodermal derivatives, and the same growth factors will cause a stem cell to differentiate into neurons. We know that from published data. So, how will the cell know what to differentiate into in terms of an environment, and why will you not get inappropriate differentiation when you put a cell back in the brain? I think they're important issues that we need to design specific tests for.

But these are beyond the scope of what we can do because we don't have a specific cell that we're going to think about. So, I'm going to just try and recast these

issues in terms of tests that one might possibly consider and leave that open for discussion.

We have source control. I know there are these privacy issues and we have to really worry about them, but in some fashion I think we will need to know something more about the cells in terms of the age of isolation because we know there are differences in self-renewal potential.

Perhaps there might be dimorphic differences. We know parts of the brain show sexual dimorphism.

Maybe we'll need to know about the sex of the cells that we're going to transplant.

I think it's going to be quite important for us to know the region of isolation because there's clearly now a lot of data which suggests that there are regional differences in cells.

Viral testing. I think maybe in terms of the CNS, we may have to think about prion diseases, and that may be an additional test that we may want to think about rather than just sort of a standard organ and tissue transplant test that we do.

As this point has come up several times, I think sample profiling. We can't look at single antigens to classify a cell. We will need to look at multiple criteria, and perhaps we'll have to think about profiling by some sort of standard criteria for any kind of cell that

we use for transplant.

This is an issue which I think may be quite important. We are deriving lines and we are hoping that we can get these cells and we can use them for a large number of people. Perhaps soon after these cells are isolated, there should be some mechanism to deposit these as a reference aliquot that you can compare your cells with when you're going to do any kind of tests or transplant back in the brain.

In terms of a manufacturing process, I think there will be issues that we should definitely keep in mind since we're taking dividing cells and putting them back in the brain. One issue that's sort of become clear from the data that's been out there is that the response of cells changes with multiple passages. For example, with neural stem cells, at least with the EGF-dependent cells, it's clear that the degree of astrocytic differentiation is far higher from late passages than it is from early passages.

The issue of karyotypic stability has been raised before, I think that's going to be very important. Anytime you passage cells, one should think about these as cell lines and maybe we should have some measure of mutation rate. Maybe we should be looking at p53. Maybe we should be looking at some other measure.

I think we should look at telomerase activity

because it's clear, at least in human cells, it seems to be a measure of degree of self-renewal potential that these cells have.

I think we should be comparing at each passage with a reference aliquot the properties that's been defined for that population at each stage.

And I think in any culture you need to worry about viral testing and antigen testing. We should be doing that.

I want to remind people here that the other thing we need to think about is that if you take cells which have many, many sort of potential outcomes because they're stem cells -- so, in this case, they could die, they could make crest cells, they could make CNS derivatives, or they could convert from one stem cell to another cell, as has been shown -- then we should really be getting some numbers on the frequency of these sort of things happening.

So, I think we have to worry about specifications of the cells when you're ready to transplant them. Given what we've heard so far, I think the purity of the cells that you hand over for transplant is an important consideration. Is it a pure population? Is it a homogeneous population? What fraction has already differentiated? What are the detailed characteristics of

this clump of cells that you're going to give?

I think we need to know something about cell division after transplant. And the reason I think this is quite important is that if you get inappropriate cell division, it doesn't have to be a tumor, it just has to be a mass, and that's going to have effects in the brain. If these cells divide inappropriately, then we need to know that. We need to know how long they will divide for because this can have important functional consequences.

This is a point that came up earlier I guess in conversation: functional assays that reflect therapeutic use. I think if you're going to put cells back in the brain, you want to have a functional assay which will tell you that these cells actually give you that right kind of function. So, we have to really define which functional assay we will use depending on which cell type we use and which therapy indication we are using them for.

I think at this stage too you need to profile that specific lot of cells that are going to be used for therapy. We need to always compare them with some kind of reference aliquot.

This last issue is numbers in a lot. That also came up earlier. How many of these things can we do? If it turns out that each cell that we use is unique to that population, if we have to isolate cells in an autologous

transplant, then maybe we don't need to do as many different tests. On the other hand, if we want to have cells which are going to be used only in 10 patients, because that's the total number of cells we have at any time and we have to go back to primary tissue to get cells, then I think a lot of this criteria of what we're going to do will change. So, what we really need to know, when we're going to use cells for transplant, is in what numbers of patients will that particular lot of cells be used.

I'm just going to briefly go through a couple of other issues which I think are important, and that's implantation control. I don't mean to say that we should be deciding which device, but I think that what's important here is to be thinking about cells and knowing that they respond to the device in a certain way. We need to have some parameters because this is quite a critical issue in terms of the number of cells you can get into the brain.

For example, in Parkinson's when you do a transplant, the total volume that you can put in into a site through a single injection is limited. You have to make a decision as to what the suspension will be like, how many cells will that mean. If you dissociate them, should it be in clumps or whether it should be single cells. We need to know exactly what percentage of cell death there is because otherwise we won't really have a read-out which

will be reproducible.

These are other issues that have already been brought up, and I don't want to go through them. But I think that these are some things that will have to be considered at some level by the committee. Should we be thinking about noninvasive read-outs when we put in transplants? Because there are a lot of things we don't know yet. Should we be putting in some "what-if" controls? Things may go wrong. Should we have to be thinking about engineering suicide genes? Should we be thinking about how we can kill cells? Should there be some other kind of noninvasive read-out which tells you that something is going to go wrong?

I'm going to end here and I'm going to show this picture from National Geographic. It's from bridge building in a country that will remain nameless.

(Laughter.)

DR. RAO: But you can see that they know how to build bridges. They know how to pour the concrete. They know how to extend it, but clearly there wasn't an overriding control which said, look, there's a slight problem here and you probably knew about this well in advance and you should have taken care of it before.

I think the FDA's role here is exactly that. It's we know how to do all of this, but we want to make

sure that somebody looks over this and controls the fact that we don't have this slight miscalculation here.

(Applause.)

DR. SALOMON: Thank you very much.

I think because the two talks in this group are so similar, what I'd like to do -- also because lunch is looming, and in the end, if we're way off time, it's always the chair who gets all the grief -- is to go to the next talk. Then we'll stop and discuss both of them together.

So, Steven? Dr. Goldman is going to talk about the isolation, identification, and characterization of adult human neural progenitor cells.

DR. GOLDMAN: I'm a little less optimistic I think, at least less sanguine, than Mehandra in terms of what we do and don't know. I think we still don't have the parts of the bridge sorted out. I don't doubt that it's not too early for the FDA to be involved with respect to that one slide of Mehandra's that was presented for subliminal comprehension only --

(Laughter.)

DR. GOLDMAN: -- simply because there are proposals out there now to use these cells clinically. In fact, there have been trials already initiated. So, by definition, it's not too early for regulatory involvement. But I think in terms of the basic science, there's still

quite a bit that we don't know.

I'm just going to be focusing not on stem cells here but more practically speaking, looking at what the human brain actually has because that in some way has to be the fulcrum upon which the rest of the discussions revolve because unless we know what the adult human brain actually harbors, it's hard to know how to perturb or influence those endogenous neurologic processes and the cell types that subserve them.

Now, without reviewing history, there are several neurogenic populations that have been described in the adult mammalian brain. As far as back as the early '60s, in fact, neurogenesis within the olfactory bulb and hippocampus where described by Altman.

It had been thought literally for a couple of decades that these were locally neurogenic populations, and what became clear in the early '90s with the work of Marla Luskin and Alvarez-Buylla and others was that the olfactory bulb in particular -- the neurons being generated and migrating to the bulb are forming from and arising from progenitors within the ventricular zone. This is a process of long-distance migration, similar in some ways to work that Fernando Nottebohm and I had done years before that in the adult bird brain where long-distance migration of newly generated neurons from ventricular zone progenitors had

been found.

Now, the hippocampus remains a population that is largely locally neurogenic with some migration from the subgranular zone, a still undefined degree of migration from ventricular zone progenitors. That's what had been known.

Actually on the model in the adult bird brain, there are progenitors lining the entire ventricular system, and these cells are only being utilized in a few discrete loci. With that thought in mind, we hypothesized that perhaps the progenitor population of the adult mammalian ventricular zone was more widespread. This is work of Barry Kirschenbaum in the mid-1990s. We found that, in fact, neuronal progenitor populations were quite widespread throughout the adult rodent ventricular system. These are subependymal chains of newly generated neurons. Chains like this can be found throughout the ventricular system. These include mitotic and neuronally restricted progenitors, as well as less restricted, uncommitted, multipotential neural progenitors.

Using that as the conceptual model, we then looked at the adult human brain to see whether humans continue to harbor a persistent neurogenic population within the ventricular zone. For those studies, we used temporal lobes resected from adult epileptics who were

having a temporal lobectomy for medication refractory epilepsy. That provides a nice source of temporal ventricular zone ventricular wall lining.

When we cultured these in explant cultures -- actually the same types of culture methods that were established years before for canaries. The factor controlled in these processes is quite conserved across species.

In any event, we saw neuronal migration and neuronal generation from the ventricular zone explants. These are neurons labeled for several markers, MAP-2, NCAM, MAP-5. And some of these neurons are generated in vitro. The silver grains indicate thymidine incorporation from a mitotic marker introduced in vitro. So, we knew from this work that there was a population of neuronal progenitor cells that, indeed, persisted in the adult human brain.

From the work of Rusty Gage and others in the mid-1990s, it became clear that FGF was a strong mitogen for the ventricular zone population in rats. We then applied that knowledge in our own observations of the role of BDNF in driving differentiation and survival of neurons generated from these precursors to do the following experiment.

We took adult human ventricular zone explants and cultured them for several months in vitro under the

serial control of FGF-2, followed by BDNF, the idea being to drive mitotic expansion of the neuronal progenitor population and then differentiate that pool using BDNF and assuring their long-term survival.

You see networks of neurons generated in these cultures, lying upon an ependymal substrate. This is simply a high power of this sample clump. When we look at these cells viably in terms of their function, we see that this is with calcium imaging using fluo-3. In response to glutamate, most of the neurons within these clumps light up. In fact, they generate action potentials as well. So, these are functional neurons.

After fixation, we can show that they express typical neuronal markers. This is MAP-2. And most of them have been generated in vitro during the period of FGF-2 exposure which is in the first week of this culture 2 months before its fixation.

Now, this was a reasonable demonstration of the neurogenic potential of the adult human subependyma, but the numbers, really when you get down to it, aren't all that impressive. We can generate thousands of cells, perhaps tens of thousands, from a given ventricular zone sample, but that's spitting in the wind compared to what we need in terms of clinical implantation or clinical utilization.

We needed to get a better idea of how many of these cells actually existed in vivo. Luckily in the mid-1990s, a number of markers became available that allowed us to at least make guesstimates of the density and frequency of these cells.

These are two markers: Musashi protein, which was initially identified by Hideyuki Okano, which is an RNA binding protein expressed by early neural progenitor cells. Here we see in an adult human ventricular zone specimen a number of Musashi expressing cells. When we look at another RNA binding protein called Hu, which is reciprocally related to Musashi -- it's expressed upon neuronal differentiation concurrent with Musashi shutdown -- we see a number of Hu expressing cells within the ventricular wall. These are essentially the neuronal progeny of these cells.

The problem is that there aren't very many of them. They're lying in essentially a cellular monolayer within the ventricular wall, and this is the kind of tissue piece. It can be anywhere from 50 to 100 grams that we have to isolate what are essentially less than 10,000 cells from. We're starting with tissue populations, cell populations in the 5 times 10 to the 10th range, and we're trying to sort from that pool cell numbers of 10 to the 4th to 5 times 10 to the 4th. This is a level of enrichment

that is even beyond that which is required for the isolation of the hematopoietic stem cell, whether from marrow or peripheral blood.

Making the purification issue all the more difficult is that there are no truly specific surface markers for neural stem cells, neural progenitors, as we've seen from the earlier talks. So, we utilized a different strategy.

This is as dense, by way of reminder, as these cells ever get. This is a stain from Musashi.

So, to pull that relatively sparse population out, we took advantage of the known selective expression of a variety of cytoskeletal and regulatory molecules in neural progenitor cells and neuronal progenitors. These are not surface markers, but rather skeletal and regulatory proteins, for which the promoters had been identified. In other words, the genes had been sequenced in total and the regulatory sequences controlling the expression of those genes had been identified.

As our prototype, we took the promoter for T-alpha I tubulin, or a tubulin that's made within the ventricular zone by neuronal progenitor cells and also by very young neurons as they migrate. This is a promoter that we obtained from Freda Miller. We took the T-alpha I tubulin promoter and coupled it to the gene encoding green

fluorescence protein and then took the resultant T-alpha I:GFP construct and asked whether we could identify progenitor cells while they're still alive, as opposed to in fixed material after the fact. For this purpose, we used dissociated ventricular zone, transfected all the cells in the cultures with the T-alpha I:GFP plasmid construct.

The question is, who's the progenitor? It's always the cell in the middle. That's the progenitor. So, the T-alpha I tubulin:GFP construct allowed the selective expression of the GFP, the fluorescence reporter gene, in the progenitors of interest. When we followed those cells under FGF conditions, we get expansion. When we switched to BDNF, some of the cells die, but others become neurons.

Given the presence in these cells of a fluorescent marker, that allowed us to use fluorescence-activated cell sorting to pull that population out. This is a typical sort graph in this type of selection. Here we've taken the T-alpha I tubulin-driven:lacZ as a control against GFP. You see nothing gated in the fluorescent fraction. This is a typical fluorescence versus forward scattered, in other words, versus cell size type of graph.

In this plot here, with the T-alpha I:GFP transfection, we see a very discrete, very small population of cells from the adult ventricular zone dissociate. It

was a 33-year-old who's down to one-tenth of his ventricular zone population being recognizable as a neuronal progenitor pool.

This is what the cells look like after FACS. And at 1 and 2 weeks thereafter, we're seeing the maturation of neurons, all of which have incorporated bromodeoxyuridine in vitro in the first couple of days after sort. So, these cells, when they're being harvested, are still mitotic. They go on to express fully matured neuronal markers, and as we'll see, they become functional.

Now, we can pull out different cell types to find at different stages different phenotypes, stage-defined, by virtue of using promoters that are transcriptionally activated at those different stages. So, it's a powerful technique.

Here we've used the enhancer controlling in the expression of nestin. Nestin you've heard mentioned before. Nestin is expressed by neural progenitor cells. It's expressed by other cell types too, so it's not entirely specific. We've also found it's not as early as we might like for true stem cell derivation, but at least we're pulling out multipotential neural progenitor cells. It's sufficient.

So, we've used a nestin enhancer driving a basal promoter, the heat shock protein-68 promoter. What

that does is target nestin expression. This is the second intronic sequence of the nestin gene which targets the expression of the gene at two neural progenitor cells -- this was the sequence first identified by Ron McKay some years back -- regulating here a basal promoter, in turn regulating the expression of GFP. When we transfect that plasmid into adult human ventricular zone, we see a very discrete pool of nestin expressing cells. Again, it's a rather small pool; 1 cell in 1,000 here is being labeled. These are dissociates not of the whole temporal lobe, but actually of the ventricular zone, of the ventricular wall. So, the dissection itself is allowing some degree of enrichment.

Also, I should mention that the transfection efficiencies here are in the 10 to 14 percent range. So, effectively you can multiply these numbers by about 7 to estimate the endogenous frequency of these cells.

These cells are nestin cells. The nestin sorted cells, unlike the T-alpha I tubulin sorted cells, when we raise them in suspension culture in a typical neurosphere prep, they will generate neurons and astrocytes both. The blue cells are neurons, the green cells are astrocytes. These are cells that have been plated after neurosphere expansion. The nestin expressing cells are still present. So, the progenitors are dividing and at

least some fraction of them are remaining in a multipotential, still mitotic state.

Now, we wanted to see whether these cells were engraftable, whether they were functional, and whether they would achieve the range of phenotypes that we wanted after in vivo implantation.

For this purpose, we used a developmental model. We purified from the human ventricular zone the nestin:GFP defined cells. After FACS, we implanted them into the ventricular system as a transuterine xenograft into embryonic day-17-aged fetal rats. We allowed the mothers to then give birth, allowed the babies to grow up, variable periods of time, anywhere from 2 to 8 weeks, and sacrificed them, and looked for the human cells in the overall rat brain to see what those cells had become.

This is what the 17 rat looks like. This is after injection of a marker dye.

This is a typical section at a couple weeks after delivery. Here the green cells have been stained for GFP, so we're looking for persistent GFP expression. GFP actually persists for a couple weeks, even after down-regulation of the promoter expressing it. It's relatively stable in these cells. So, we're looking at double-labeled green and red. Therefore, yellow cells, which are human-derived, and the red cells are the host rat cells, rat

neurons, which have been identified by Hu. So, essentially we're generating chimeric cortices here of rat and human using the adult human-derived multipotential neural progenitor cell. So, we know these cells are competent to integrate into developing cortex.

I'm not showing the data here, but we've implanted at later time points and seen differentiation of these cells into subcortical populations that are predominantly oligodendrocytic as well. So, we do think that at least the nestin defined pool is multipotent.

Now, it's still a relatively unusual population, the ventricular zone pool, unusual in terms of numbers. So, we wanted to look at something a bit more abundant and something that might be a bit more active in vivo.

This is a slide of Rusty Gage's two years back where he and his group identified mitotic populations in vivo in the adult human hippocampus. This is a BrdU-labeled granule cell neuron within the adult hippocampus. So, we focused upon the hippocampus with the same types of protocols, taking normal hippocampus, dissociating it, and in fact, we were easily able to demonstrate mitotic populations of cells that gave rise to neurons. That in itself wasn't a surprise. We found that the cells were able to drive the T-alpha I tubulin promoter and

differentiate as neurons. So, that gave us the technical wherewithal to be able to sort that population. We utilized exactly the same protocol now as we did with the ventricular zone, taking out normal dentate gyrus.

Now, I should mention actually for the clinicians among you that the ventricular zone preps, by and large, were down with patients with medial temporal sclerosis who were having temporal lobectomy for that reason.

These cases were patients either with focal cortical epileptogenic foci. They required corticectomy and subtemporal lobectomy despite normal hippocampi or were non-epileptic patients who were having decompressive lobectomy in the setting of trauma and a couple of aneurysmal resections.

So, this is normal hippocampus. Dentates dissociated, T-alpha I tubulin is transfected into it. We wait for expression and then FACS the sample.

Basically this is the population that results. This is 4 weeks after FACS. Pure populations of neurons generated from the hippocampal progenitors. This we believe corresponds to the population that Rusty had originally identified in vivo.

The numbers are between 10- and 20-fold higher for a given dentate gyrus sample relative to a given

ventricular zone sample. So, there are a lot of these cells in the adult human hippocampus.

We've looked at patients as young as 5 and as old now as a woman in her 80's, and we see very little fall-off. This is simply comparing a 5- and a 20-year-old. But all the cases have shown FACS sorted pools of at least 1.1 percent of the population. Again, taking into account the transfection efficiency, we're looking at potential progenitor populations of at least 7 percent of the total dentate gyrus, which is really extraordinarily high. It suggests either the presence of an abundant population or, for that matter, of a persistently mitotic dentate gyrus granule neuron phenotype, in other words a neuronal phenotype that's potentially competent to dedifferentiate to a mitotic pool.

These cells are functional. This is the work of Maiken Nedergaard after loading with fluo-3, the calcium imaging dye, and the same population after glutamate exposure. The cells all light up. These represent at least 4-fold increments in cytosolic calcium in response to glutamate, typical of voltage-gated calcium channels in mature neurons.

You can actually target the GFP-expressing cells in vitro and record from them. This is a patch-clamp analysis of Jian Kang. This is in a voltage-clamped

configuration. We see current injection related current increments, very typical again of the fast sodium channels of neurons.

Now, I'd mentioned before that we can target essentially progenitor populations of interest at will, as long as we have a promoter or regulatory sequence that allows us to identify that pool. When we culture adult ventricular zone, we see not only neurons generated, but also oligodendrocytes.

In rats, the oligodendrocyte or at least the glial progenitors have been looked at for many years, and it's a very highly abundant pool and a very active pool.

In the human, it's been a much more controversial story through the years. Adult human oligodendrocytes appear to be every bit as postmitotic as neurons, and yet oligodendrocytes are generated to subserve remyelination in a number of acute demyelinating diseases. It's never really been clear where those cells are coming from. A number of attempts have failed at isolating human oligodendrocyte progenitors, it turns out, because some of the markers really are different in the human population.

In collaboration with Peter Brown and Michelle Gravelle in Montreal, we took the early promoter for cyclic nucleotide phosphodiesterase. This is a protein that's made by oligodendrocytes but also by their progenitors.

There's a segment of the promoter of the CNP gene that is expressed differentially in the oligodendrocyte progenitor. That was work that Peter Brown had established through the early '90s.

So, we were able to take the CNP-2 segment, this early segment of the CNP promoter, couple it to GFP and then transfect adult human white matter. So, these are white matter dissociates. The question once again is, who's the progenitor? For that matter, in this case, we didn't even know whether a progenitor existed. So, this was a bit empiric.

And these are the cells that express the CNP-2 driven GFP. They're very, very small bipolar cells. In fact, it would be very easy to lose in a culture otherwise. Very small, very undistinguished.

When we sort them -- actually it's a fairly large number. We're not showing it here, but this is about four-tenths of a percent of the adult white matter dissociate, translating to about 3 percent of the white matter pool. These cells at the time of harvest are almost entirely mitotic. It's an extraordinarily mitotic pool after they're removed from the brain. At 6 hours and 24 hours in vitro, they are all incorporating bromodeoxyuridine and are expressing at the time of harvest the A2B5 antigen of the GQ ganglioside, which is a marker

for oligodendrocyte lineage cells. But many of the other markers that have been used for identifying oligodendrocyte progenitors in the rat brain, particularly O4/O1, are not expressed by these cells at this point. They're solely making A2B5 among the early oligodendrocyte markers.

When we follow those cells over 2 weeks, 3 weeks -- we've taken them much further out than this -- they go on to develop mature oligodendrocytic antigenic expression. We can show that those oligodendrocytes generated in vitro were, in fact, generated mitotically. They've incorporated bromodeoxyuridine in those first few days in vitro. We can actually see layers, carpets really, of purified oligodendrocytes generated from these cells.

Starting with a couple of grams of adult human white matter, if we pull out 10,000, 15,000 CNP-2:GFP defined progenitors, we typically have at least 10 to the 8th after a month in vitro. So, we can expand this population considerably generating relatively pure populations.

Now, before moving on to the utility of these cells, I just inserted this in the break. With regards to the conversation before, the issues arose with regard to the lineage restriction of these cells. Well, this is a parenchymal progenitor. By any criteria that we can use, it's biased very strongly towards generating

oligodendrocytes. In basal culture conditions, 93 percent of these cells will generate oligodendrocytes. We see only 7 percent astrocytes and essentially no neurons at a month in vitro.

But when we played the cell sorting as a function of cell density and plated cells at different densities, we found that when we went to very, very low densities, post sort -- so, now we're dealing with a highly artificial situation of very, very pure cells of a given phenotype at very low cell densities -- 10, 100 cells per ml, no greater -- we saw a diversification or a degradation, depending upon your point of view, of phenotype. So, now the CNP-2 defined cells started to express both neuronal and astrocytic markers. In fact, we can generate neurons and astrocytes, as well as oligodendrocytes, quite freely in a density-dependent fashion. It's only at relatively high sorting densities of at least 5,000 cells per ml where we see essentially an oligodendrocytic phenotype exclusively being generated.

So, these cells, even though they're parenchymal and in the adult human, actually have quite a bit more in the way of phenotypic potential than we had realized. They may have every bit the degree of phenotypic potential as their ventricular zone counterparts. It's simply that they're being restricted by the regional

environment in a bit of a harsher way.

Now, obviously, we wanted to see whether or not these cells were engraftable, whether they were potentially utilizable. Martha Windrom in the lab established a lysolecithin lesion model. This is a standard technique for demyelinating adult brain. We modified a technique of Jim Goldman by putting lysolecithin -- it's a detergent that causes reversible demyelination -- in as a very slow infusion bilaterally into adult rat brain and allowed the detergent to very slowly diffuse in a longitudinal fashion, to follow axon tracks in the corpus callosum. And by putting injections at the same depth level bilaterally, we end up with these centromedian plaques that are in the depth of the corpus callosum. It provides a very nice model for reversible demyelinating placque lesions of the adult nervous system.

Now, we injected the adult human oligodendrocyte progenitors. Now, these cells -- this was early in the process. These were simply di I labeled. We looked at a week and saw that the cells -- here's the injection point -- migrated within the lesion, very, very rarely beyond that. So, they're following cues to stay within the lesion. They migrated extraordinarily. Here's a midline. It's a bit off kilter photo. But this lower power montage, you can see the cells migrating right across

the midline right over to the other hemisphere. Again, they're remaining within the lesion. We see some departure from the lesion to follow blood vessels, here tangentially, here coronally, but otherwise, the presence of normal intact myelin seems to be nonpermissive for oligodendrocytic progenitor migration, which for these purposes is very adaptive.

Now, to try to make these cells again more useful, we wanted to take advantage of their abundance -- this is a far more abundant population than the ventricular zone or the hippocampal pool -- and at the same time not lose the 80-odd percent that we were losing up front by virtue of transfection inefficiency. Since we had identified A2B5 as being expressed by these cells, we sorted on the basis of A2B5, looking for A2B5 expression by these cells as a sufficient marker for their extraction.

This is a young brain where the percentages are a bit higher than in the normal adult. But here you can see seven-tenths of a percent of the population of the actually juvenile white matter expressing the CNP-2 driven GFP after transfection, while almost 8 percent expressed A2B5. Now, in the adult, our average is now just over 3 percent of the population by A2B5. So, we can increase our yield substantially.

We took the A2B5 sorted cells and injected

those into the adult lysolecithin model. Here's a control at 2 weeks. The lesion tends to remain a lesion for roughly 5 weeks with this model.

This is another animal. Here is a corresponding region after engraftment and migration of the adult human-derived progenitor.

The red stain is for CNP protein, the early oligodendrocyte protein. When we look at the green cells -- and here we're targeting the human cells using an anti-human histone that is specific for recognizing human cells. Vis-a-vis the issue that came up before with regards to recognizing human cells in the rat context, we have a few ways of doing that now.

The other that I'm using interchangeably here is in situ hybridization for human alu sequences.

In any event, looking at the double-labeled cells here, we can see that the human cells are starting to make CNP protein. In fact, within a week thereafter -- this is now 3 weeks after the lesion, 18 days after the implantation -- they're starting to make myelin basic protein as well. The red stain here is for myelin basic protein, a mature myelin protein. You can see within the lesion bed, there's diffuse expression of MBP. When we confocal and actually stain for a human-specific MBP, we see myelin basic protein expression by these clumps of

human oligodendrocyte progenitors in the engraftment model.

Now, there's still a lot we don't know. We don't know whether this remyelination process is functional. We do know now that the axons are being enwrapped, but we don't know whether or not compaction is appropriate. We don't know whether or not these are physiologically functional remyelination processes that are occurring. That's work underway now.

Now, I just wanted to close. Lest an alternative not be mentioned at the meeting at all, beside implanting cells, implanting stem cells, implanting lineage-restricted progenitors, I think it's important for us to be able to utilize the endogenous progenitor populations that are present to understand their role in disease, what happens to them in disease, where they may contribute to disease, where they may contribute to endogenous or spontaneous recovery. One way to approach that is to use the very limited knowledge we have at this point in terms of the growth factor control of these cells to try to induce endogenous progenitor pools to be neurogenic and in fact to be neurogenic in otherwise non-neurogenic regions.

Now, there are a number of ways to tackle this. This is one approach that we used as a result of a collaboration with Ron Crystal's group at Cornell. We took

an adenovirally delivered, in this case, just a marker gene, GFP driven under CMP promoter control, and injected it into the ventricular system to see, as a first level of analysis, where an adenovirally introduced transgene would get to after ventricular injection, the logic being to try to turn the ependyma or the ependyma/subependyma into an endogenous source of secreted neurotrophin introduced by the viral transgene.

Here is simply a low power sagittal section of the adult rat brain after the adenoviral GFP introduction, and you can see that the tag is remaining restricted to the ependyma and to a much lesser degree the subependyma. There's some immigration of cells into the corpus callosum, but nothing into the gray matter parenchyma of either the striatum or the cortex. So, we know that if we give an adenovirally delivered transgene, that the adenovirus itself and the cells expressing those transgenes will remain restricted to the ependymal surface.

So, with that logic, we constructed an adenoviral BDNF vector that has GFP and IRES association. So, it's expressing both a marker and BDNF. BDNF we and others have described as, again, a differentiation survival factor for neurons generated from the endogenous ventricular zone progenitor pool.

Here's the virus. We used a protocol of serial

injection with bromodeoxyuridine for essentially 3 weeks, followed by sacrifice 3 weeks following virus injection. So, these animals are producing high levels of BDNF in response to the viral injection, which is restricted to the ependymal surface again. This is in situ hybridization showing BDNF and GFP message against the control. Nothing in the parenchyma. The idea again being that the ependymal cells are making the BDNF. The subependymal progenitors are then being exposed to the BDNF without being infected by the transgene or infected by the antivirus, rather, themselves.

That results in very high levels of BDNF expression both parenchymally and here in the CSF. We're looking at a couple of nanograms per ml, which approximates what we actually had in vitro to study these cells. So, the CSF is achieving very high BDNF levels.

In response to that, we see a tremendous increase in neurogenesis to the olfactory bulb. Remember, the olfactory bulb is undergoing neuronal recruitment normally in an ongoing fashion from the cell population. Now, if one looks at the olfactory bulb, you can see double labeling for the neuronal marker in red, beta-III tubulin, and the green indicating bromodeoxyuridine incorporation. These cells are being recruited as neurons, here double confocals for beta-III tubulin and MAP-2.

This is what the comparison of the BDNF versus a null injected set of animals shows. If one does a low power camera lucida reconstruction where every dot represents a BrdU incorporating neuron, you can see a substantial increase in density of neuronal recruitment, neurons being added to the BDNF-treated olfactory bulb. So, we're inducing neurogenesis from the endogenous pool here.

This is what it looks like quantitatively, BDNF versus an AdGFP, no BDNF insert control. We're looking at numbers here, several thousand newly generated neurons per cubic millimeter, so very high recruitment numbers in response to BDNF.

Now, the issue that actually I want to close this with then becomes can we use this approach to cause neuronal recruitment in regions that are otherwise non-neurogenic. So, we scored the addition of newly generated neurons in the cortex, the striatum, the septum, as well as the olfactory bulb in these animals.

I should mention that we actually saw very low degrees of neurogenesis in the cortex in response, too low to count, but is there. We saw nothing whatsoever in the septum. But in the neo-striatum in the caudate putamen analog, we saw very high numbers and densities of newly generated neurons in the striatum and only in the BDNF-

treated animals.

These are simply confocals where we've serially reconstructed Z-dimension stacks. The red is a neuronal marker, beta-III tubulin. The green is bromodeoxyuridine. This is simply to be sure that the newly generated cells as marked by BrdU really are neurons, that they're expressing neuronal markers throughout their extent.

When we look at this quantitatively, we're generating on average 140 neurons per cubic millimeter within the neo-striatum. This is a fraction of what is generated in response to the AdBDNF factor in the olfactory bulb, but nonetheless, it's really a significant addition. If one accumulates that number over time -- Yuesh Melniki in the lab has done this stereologically in a large sample at this point of rats looking at total striatal number and the newly generated fraction thereof -- we can predict essentially a 10 percent replacement of the normal adult rat neo-striatum every couple of months.

What's particularly interesting here and germane to this morning's discussion is that these appear to be functionally relevant, or at least functionally relevant, neurons. The striatum is a gemisch of different phenotypes, but the cell type, in many ways, of the greatest interest to neurologists, at least, as a target of disease is the medium spiny neuronal population, which is

largely a striatal/pallidal projection pool as well as containing some striatal/cortical cells.

Now, the medium spiny population is, of course, the population lost in Huntington's disease. It's characterized by expression of a number of markers, the calcium binding protein calbindin, as well as GABA and its synthetic enzymes. We found that the cells being generated in response to the AdBDNF factor express almost exclusively calbindin. Most of them are GABAergic. They don't make any DPH or acetylcholine or the other markers of striatal interneuronal phenotypes. So, we think that the cells being generated correspond to the medium spiny neuronal pool. In fact, this is work we're now doing in Huntington's mutants to see whether or not we can, on any level, not necessarily restore function, but at least prolong the degree of life, the life expectancy of animals that develop the disease phenotype.

I think for this audience, the take-home points are unnecessary. The real bottom line is that we have a number of different lineage-restricted pools in the adult human brain. Some of them are multipotential. I say they're multipotential and lineage-restricted at the same time in that we have not shown that they are competent to generate non-neural phenotypes at this point. So, these are relatively restricted, as least as defined by nestin

and, for that matter, other early promoters that we've used, relatively restricted neural phenotype.

Then we have more restricted populations vis-a-vis the T-alpha I tubulin defined neuronal progenitor pool, the CMP-2 defined oligodendrocyte progenitor pool, that nonetheless may have some transdifferentiation or diversification capacity under the appropriate conditions.

The abundance of these cells is variable. It varies by age. It varies by region. We still don't understand the lineage, the lineal relationship or the genealogy of these cells with respect to one another.

It's fair to say that the most abundant progenitor pool of the adult human brain we've been able to identify is that of the adult white matter. Whether we can use that pool for purposes other than remyelination still remains to be seen.

Again, not to forget the viability of approaches directed towards inducing the endogenous progenitor pool and, for that matter, to combine these approaches to potentially induce endogenous progenitor pools in the context of exogenous progenitor implantation or co-implantations.

Thanks for your attention.

(Applause.)

DR. SALOMON: Thank you very much.

Well, I think just so that we have some sort of framework, I'd like to have some discussion of these two very important talks. We're also a little bit off time. What I'd like to do is have about a 10-minute discussion of this, which isn't going to adequately cover it, and then break for lunch, which will be about 12:30. Then instead of restarting the meeting at 1:05, we'll restart at about 1:30.

So, what I'm opening up for discussion is Dr. Rao's and Dr. Goldman's talks. I took notes with some questions that I wanted to ask.

One question I had for Dr. Rao was that he brought up this idea that the different precursors could be defined by different growth factor dependence. So, you had FGF responsive and EGF responsive and FGF and EGF. So, the question I have for you is how different are these different populations. Are they possibly overlapping, or they actually just unique, distinct populations? Then it gets quite complicated, as you said.

DR. RAO: It's not our data, but I think Dr. van der Kooy's lab actually did a series of very nice experiments which showed that these are distinct populations. They used actually chimeras from FGF receptor knockout animals and showed that these two cells overlap in developmental time, but they're clearly distinct and you

can actually isolate both of them at certain specific stages and at early stages isolate one.

The knockout data from FGF receptor knockouts and EGF receptor knockouts also clearly shows that at early stages in the ventricular zone, there's only an FGF receptor dependent cell, and at later stages, there might be an EGF receptor cell.

So, both of those say that they're clearly overlapping in developmental time and that two precursor populations, at the very least, exist.

In terms of functionally their being different, I think there's quite a lot of data in terms of the frequency of neuronal differentiation at the very least. So, the FGF dependent cell seems to have a much higher frequency of both after the short-term and long-term culture into making neurons, while the EGF cell seems to make at a lower frequency. The phenotype of the neurons is different. So, mostly what you get from EGF dependent cells are GABAergic sort of interneuron phenotypes, while what you get from the FGF dependent cells are really these sort of much larger glutaminergic type of neurons.

DR. O'FALLON: Professor Rao had a list -- I think it filled up the page -- of potential neurologic problems which we might be trying to address. At top the list were cerebrovascular accidents. Ischemic strokes come

in a variety of different environments. 75 percent of people with those accidents will be hypertensive. Some substantial number will have diabetes. A whole bunch of them will have had cardiovascular situations. Are we going to need different cells in every one of those contexts? And does the same example persist for all of the other problems that you raised?

DR. RAO: I think for all strokes perhaps you might need certain classes of cells, but it will also I think depend on the region involved. I think, for example, brainstem strokes will be very different from cortical stroke in terms of therapy. I'm absolutely convinced of that.

DR. REID: I wanted to make three comments and questions. One is in other fields it's been shown that when there's very striking density-dependent effect on cells with the same growth factors, the most common matrix molecule that's involved in that are the proteoglycans. The proteoglycans are probably the major or one of the major matrix molecules of the brain. So, there are a number of groups who have done a lot of work on identifying the particular proteoglycans that are in the brain and analyzing facets of their functions.

So, one prediction would be that if you take your cells at the low density but add a proteoglycan that

is known to be generated by the cells at high density, that you would get the same result. If that's the case, then proteoglycans could be one of the factors that you might want to utilize in order to drive cells in one particular way.

The second issue is one raised about the immunogenicity. Certainly in a number of fields, particularly in the pancreatic islet field, they have made use of fetal pancreatic islets to try to overcome immunological rejection phenomenon. What they found was that they could actually buy some time when they would have not a significant amount of rejection phenomenon in the patient, but with time, those cells acquired the immunogenicity that adult cells would have.

We have just done the same thing in the liver stem cells. Hepatic stem cells have virtually none of the immunogenic markers that might elicit an immunogenic response, but they very quickly differentiate into cells that do.

So, I think we're going to probably have to adopt the procedures long used by the hematopoietic field in which they tissue type the cells, and that's probably how we'll have to overcome immunological rejection phenomenon.

The last point is that at least in the liver

one of the critical variables on the extent of expansion of the cells is whether there is a cellular vacuum. That is, it turns out to be in the liver the majority of the cells are polyploids, they're tetraploids. I don't know if the same phenomenon exists in the brain. But you have to eliminate a significant percentage of those tetraploid cells in order for donor progenitor cells to expand significantly.

So, it means for us that, when we start to treat, for example, children with inborn errors in metabolism where the they have an intact liver acinus, we will probably have to give higher numbers of progenitor cells in order to get a requisite response. Whereas, if you have a patient with liver failure, in many cases it's most commonly due to loss of the polyploid cells. You can probably inject smaller numbers of cells because they have a greater expansion potential. So, the bet would be that in the brain, the same phenomenon may be true.

DR. RAO: Maybe I'll answer the last part and see if you can tell me whether you agree.

So, Steve showed these cells which were A2B5 positive and he transplanted them in an intact brain, and what you see there is that they migrate quite extensively. So, it seems to be dependent on the cell type that you put in and what its behavior will be. So, it may not be as

difficult as the liver where you necessarily have to lose a large number of cells before you see differentiation.

But by the same token, Steve pointed out that the cells seemed to be located in that part where there was injury, so that they were in the lysolecithin lesion, for example. That's true even for neurons. When you have a cavity, then you will see that the scar will look like the cell.

So, certainly both issues may be true. We just don't know enough to be able to make a clear-cut prediction of what each cell type will be doing.

Your point on the matrix and density is also very well taken. We know, for example, that the effect of FGF is modulated by heparin, and heparin cells make proteoglycans. In fact, when you grow them in culture, if you add that, you can reduce the dose of the cytokine 10-fold.

It's also true that there are several other inhibitory proteoglycans which are quite critical in their response in terms of migration. So, there's chondroitin sulfate proteoglycans which are also quite critical. So, that point is absolutely true both in culture and in vivo.

However, I also think we just don't know enough about all of these extracellular matrix proteins to use them in the way you suggested in terms of being able to

direct differentiation in any fashion.

DR. NOBLE: It seems that one of the things that's already clear from this morning is that the pace of discovery in the biology of the cells is very different from the actual pace that will be followed in clinical trials because while we discuss this complexity, the fact of the matter is that each individual group or company that's going to move into clinical trials has its particular population in which it has an invested intellectual property position, and it doesn't have the freedom to choose from this great panoply of cells and say, well, which one is the best. They're going to go forward with what they have.

So, one of the things that I thought was very important in Dr. Rao's presentation was this list of information that perhaps needs to be collected in every clinical trial about the kinds of cells that are used so that at some point in the future, where we're able to make sense of this great complexity, we actually have this information to go back to rather than to have it be done in a slipshod manner that these things appear to sometimes be being done.

DR. SALOMON: Steven, when you started off, you actually introduced yourself saying you're not quite as sanguine as Dr. Rao. I was waiting for the non-sanguine

part of the talk.

(Laughter.)

DR. SALOMON: You ended up using adenoviral gene therapy.

(Laughter.)

DR. SALOMON: I'm thinking this guy is pretty optimistic on my part.

DR. GOLDMAN: Adeno, of course, was intended as proof of principle only.

(Laughter.)

DR. GOLDMAN: Nonetheless, I'm differentiating between the basic biology and the clinical utilization thereof. I think that's obvious.

One thing that I thought was a take-home point of this morning's discussion was that all of the debate, with regards to the relative priority of importance of understanding lineal relationships, the microenvironment, the interactions between the two, ultimately will be meaningful only in the context of discussion surrounding each disease choice, each disease target. The priorities that obtain in designing therapeutic strategies will very much be a function of what disease target one is trying to use these cells for, "these cells" being a very generic term in and of itself. I don't see any of the disease models yet being at a point where we can move very quickly

with any assurance towards clinical trials.

I've focused a bit in my own work on the demyelinating diseases simply because, in my simplistic way of thinking, those are the most simple and will be the most readily addressed. I think when we start thinking in terms of stroke, traumatic brain damage, at least as a neurologist, I'm very pessimistic in terms of utilizing any single cell type towards any meaningful treatment, at least at this stage in our current ignorance. It's going to be years before we develop the degree of understanding that will allow us to do so safely, at least again in my own opinion.

Whereas, with some of the more phenotype-defined and phenotype-specific diseases such as the acute demyelinating illnesses, we may be able to at least attempt therapeutic trials, recognizing that we still have a substantial degree of ignorance but also working on the assumption that we're likely to do very little harm in trying to address these more simple problems.

But the sanguinity comes from contemplating Mehandra's list of diseases, and thinking in terms of meaningfully treating any of them at our current level of understanding I think is premature.

DR. RAO: In my defense --

(Laughter.)

DR. RAO: -- I'd just like to add that, in general, whenever I've given a talk like this, people have told me I'm too pessimistic. I didn't take that list as a specific indication of therapy. I just abstracted the list from Harrison's Textbook of Medicine --

(Laughter.)

DR. RAO: -- simply to point out that this is the list of neurological disorders in which people have said that they would like to use stem cells in therapy. Certainly I am not suggesting that we should use stem cells in any of these things.

DR. GAGE: Listening to this morning and this talk and then hearing what the topic of the conversation is, stem cells, at some point we should have some discussion about really are we talking about stem cells. Is it really useful to even talk about stem cells if we're talking about cellular therapy? Because in a broader sense, we're looking at harvested cells that have therapeutic value. While there's, I think, a lot of attention surrounding the conceptual problems associated with the term "stem cell," I'm hearing in the talks that it's more important to define the cell type that's appropriate for the disease and you may isolate the cell at different time points depending upon when it's needed.

One of the key features that separates out, it

strikes me, this idea of stem cell progenitor population from what's been going on in fetal tissue grafting for a long time is the ability to propagate the cells, this ability to propagate the cells in some immature state so that it can retain the capacity for differentiation down the lineage that one is interested in. Whether or not you're propagating it was a stem cell or as a totipotent, pluripotent, multipotent or progenitor cell doesn't really make any difference from the perspective of the clinical application so much as that you can retain the plasticity of the cell so that it can effectively and efficiently generate the cell type that will be effective down the road.

I think sometimes this idea of having to fall back on the concept of stem cells and the definitions associated with stem cells is a bit of hindrance in our ability to maybe think about applied problems associated with cell therapy.

I was wondering if the speakers had any -- since they were talking about lineage-restricted cells.

DR. RAO: I think the emphasis was more a function of classification of what the cells can do when you call them lineage-restricted or we call neuron-restricted or biased because that is of some predictive value in which cell you use. It's a function of

classification. That's really critical. So, I absolutely agree with you that you really need to know what the cell can do and what it's properties are going to be when you use it. That's why you classify them in some fashion.

DR. GOLDMAN: Well, there are operational consequences to that. To the extent that there's an inverse proportion between the profligability and the lineage restriction, which at least has been the case with most of the models thus far presented, thus far published, we may find difficulties in terms of harvesting -- I think this is axiomatic. It's not surprising -- lineage-restricted, more functional, more competent lines that can be maintained as lines. I think that's the situation. We're getting back to the earlier discussion. We're going to be the most dependent upon going back to new source material.

DR. CHAMPLIN: Just reflecting that one needs a stem cell deficiency disease ideally to test this, the neurologic equivalent of aplastic anemia, and at least in the hematopoietic systems, if you treat stem cell disorders like myelodysplasia just by giving identical twins stem cells, of course, you don't cure those patients because the disease itself is suppressing the growth and differentiation of the normal stem cells. So, in these situations, your endogenous stem cells are being suppressed

and/or you've reached a regulatory state where the nervous system no longer is providing a proliferative stimulus at the stem cell compartment. So, one needs to modify the local microenvironment to allow the stem cell transplant, if you will, to proliferate and restore neurologic function.

DR. GOLDMAN: Again, that's a function of disease. It's very likely that there are diseases where the stem cell pool is functionally knocked out and others where it's very likely to be adaptively involved. Unlike the hematopoietic system and unlike hematology, I think the problem in neurology is that we simply don't even know what the diseases of stem cells are, or at least of the diseases that we might presume to exist of resident neural progenitor cells, not to say stem cells. The fact that these progenitor populations exist and are abundant suggests that there is very likely some pathologic manifestation in disease of their dysfunction, and we don't even know what they are yet. So, again it's premature to draw that parallel I think to the hematopoietic system.

DR. MULLIGAN: Just to go back to Rusty's point, I was struck by John's talk when the stem cell-ness of his cells was looked upon as almost a manufacturing concept. So, I think that one very simple concept is how do you make a batch of the cells.

But I think the uniqueness of the stem cell approach is definitely the migration capacity, the trafficking capacity. Although, while it's very true that the natural environment doesn't occur, certainly from our work from, say, endothelial cell specification after bone marrow transplantation, there is such exquisite trafficking in response to bone repair. So, for instance, if you ligate a coronary vessel, after doing a bone marrow transplant, the only situation where you'll see endothelial cells is right in the infarction zone.

So, I think that we should keep in mind that, in addition to what I would call the simple kinds of applications, which is the just manufacture and amplification, that understanding, albeit in an abnormal, maybe wound-healing context, these stem cells, depending on exactly what they are, may have very different kinds of properties.

I had a gene therapy question on the adeno case. It gets back to the hematopoietic system, the fact that when you treat animals with 5-FU, which kills off a lot of cycling cells, you see a rebound in terms of cells that have some sort of reconstitution potential. Is it possible that the mechanism of the AdBDNF is more destruction, adeno-mediated destruction of those cells, with perhaps BDNF providing some protective effect over the

destruction? I noticed that from the bar graphs that you had, it looked as if even the AdGFP seemed to induce some increase in the proliferation.

DR. GOLDMAN: In the striatal neurogenic population, that's right. That's why we did that control was to see whether or not there was an adeno-mediated cytokine release.

What we've actually parenthetically observed in adeno-dependent endothelial production or in adeno stimulation, it consisted of endothelial production of BDNF itself which would directly relate to your question.

I don't have a straightforward answer there in that we have done a no adeno control and see a smattering of striatal neurons being added. That surprisingly is not new. A number of groups through the years, as far back as Caplan in the '70s -- van der Kooy had a couple of reports in the mid-1900s on this -- in the context of looking for -- in various controls looking for neurons being added to adult brain, at least in the rat, there are consistent reports of occasional neurons being generated and recruited to the adult neostriatum.

So, here we see an adeno BDNF associated increment, a substantial increment, in neurons being recruited to the neostriatum. We see a very, very low number of neurons being generated and recruited in the

adeno no condition. Those numbers are so low that I don't know that they're any different from that which we would see in the absence of the adeno.

So, the quick answer is I don't think that the adeno per se is affecting the natural history of these cells. I think the more important issue here is whether an adeno-dependent cytokine effect might be acting synergistically with the BDNF.

DR. MULLIGAN: Yes, that was really what I was getting to, but even whether the BDNF was acting synergistically with the adeno.

DR. SALOMON: One last question.

DR. DRACHMAN: Dr. Rao raises a real paradox, that is the more restricted the neurons, the safer, the less restricted, the more they're capable of doing a variety of things. From the point of view of a neurologist, it's always worth remembering the brain is not a liver.

(Laughter.)

DR. DRACHMAN: Santiago Ramone y Cajal and others pointed out how many thousands of different types of neurons we deal with. We as neurologists live on the notion of selective vulnerability, that is, all these diseases which pick out tiny groups of neurons that characterize the disease itself. So, the attractiveness of

stem cells, meaning the tremendous capability of doing almost anything, is sort of contradicted or balanced by your need for defining what they are and using just the right ones.

DR. SALOMON: When you have this many intelligent people with an interesting subject, it's never a good time to stop but I think for biological reasons --

(Laughter.)

DR. SALOMON: -- that are short-term rather than long-term I think we'll stop here. See you all at 1:30.

(Whereupon, at 12:50 p.m., the committee was recessed, to reconvene at 1:30 p.m., this same day.)

AFTERNOON SESSION

(1:48 p.m.)

DR. SALOMON: The one thing I wanted to do this afternoon, just because I think that the discussions that we're having -- and I've gotten feedback from a number of people at lunch -- are really excellent and where a lot of the meat of what we're going to provide the FDA in this is going to be -- is to just ask the speakers to try and stick to like a 25/30-minute talk more than the 35-minute/40-minute talk, which allows us to stay a little bit more on track because I really do hate cutting off these discussions, as they're really I think very, very valuable. So, usually somebody decompensates whenever I say that. It's, oh, my God, I've 10 slides too many.

The first talk of this afternoon is Jeremy Sugarman from Duke University, Anticipating Ethical Issues in Clinical Experiments Using Stem Cells.

DR. SUGARMAN: Well, I appreciate the opportunity to think about these issues with you, and it is always, I think, important to bring the ethics in from the beginning of when deliberations about thinking about moving forward to clinical experiments with people are being contemplated, to think about the ethics early rather than waiting for something wrong to go on or to try to address what might be a political or a moral debate.

I want to thank especially Don Fink for his help in getting materials ready for this meeting in preparation for it.

Starting this, even though I do have a Power Point presentation, I have serious slide envy here. I don't have an ethics gel or I don't have any ethics fluorescence.

(Laughter.)

DR. SUGARMAN: I really am feeling inadequate to this task. I could build on the word "potency" but I won't.

(Laughter.)

DR. SUGARMAN: So, I'll just move forward with what I hope to do.

What I'd like to do is give you an overview about the focus and background conditions of the area of ethics that I'm going to address, and then talk about what are some of the issues that might be important when we move from bench to bedside or thinking through that, and then some additional considerations that are going to be of relevance when thinking about cellular stem cell therapies.

The focus of my comments today are going to be on clinical trials, not the collection and storage and all the wonderful things people do to stem cells. I think a lot of these issues have been discussed, debated,

deliberated, created lots of emotional statements on either side. There are some critical issues at hand, but there's plenty of scholarship out there and plenty of places to go for work in that area. I think we're at another threshold here and those are the kinds of questions I want to take on.

In addition, I don't want to try to tackle what the issues might be for using stem cells for anything, from diseases outside the range of neurologic disorders. I don't want to go into the treatment of cardiac disease or all the other chapters in Harrison's. What I'd like to do is just focus on sort of the candidate diseases here, staying away from the use in angst which I think would be useful in the future. Parkinson's disease, amyotrophic lateral sclerosis, ALS; and spinal cord injury.

I'm also going to make an assumption about adults as potential subjects rather than kids. Basically a spinal cord injury obviously can happen to children. It raises different issues for consent and proxy decision making, which we can talk about if it becomes an issue, but I just don't want to complicate it at this point.

Here are background conditions that I think are of great relevance. There is a huge amount of trust in the scientific enterprise and its oversight. Despite the scandals that take place from now and then, all the

empirical work in ethics shows that folks trust us. They trust investigators. They trust institutions and they trust the oversight mechanism as a whole to get this right. There are folks who don't trust us, but when you talk to patients in hospitals around the country, they trust us.

The trust, when you hear it expressed through a variety of empirical work, is very humbling. The expectation of looking at somebody in a white coat taking care of someone who's sick is a very powerful symbol that clearly can't get dismissed. And the thought about what it means to get this right becomes magnified for those of you that have the honor of working in the clinical setting.

At the same time, there are huge arguments for access to investigational drugs. This debate got started, obviously, with the AIDS epidemic and clamoring and changes in drug approval mechanisms and approval processes. And especially for devastating disorders, it's hard to argue in the face of folks who are suffering and saying we want access to this. We don't care if it's going to hurt us. We're dying, we're suffering, we're in pain. Whatever the condition is, it is really hard to say we're not ready to give you something. We just don't have it. It's difficult.

The scientific enthusiasm is enormous. We saw some great science this morning. I'm sure we'll see some

more great science this afternoon. Scientifically these are great ideas. Gene transfer experiments, sometimes called gene therapy, exciting ideas, novel. They build on what we assume. We like it. So, the science here is high.

The financial and moral stakes here are high as well. It's no kidding that there has been a lot of money expended on doing this science. The moral stakes of messing with folks and messing with people's wallets and lives and things like that are pretty high as well. So, it's important that we get this right, especially in light of all the trust.

That's sort of the preaching part about ethics. Let me just switch gears here and give you some principles.

We have well worked-out principles once we get into clinical research. The Belmont report that came out of the National Commission for the Protection of Human Subjects and Biomedical and Behavioral Research -- there's no test on the name of that commission -- outlined principles and outlined rules that we have once clinical trials start.

But what goes unaddressed is the really crucial step of doing things in people for the first time. It's not to say deliberations don't go on, but they're important.

I'm going to draw here on an article that I did

in Science a few months ago that lays out the rationale for these principles. After lunch, I'm not going to try to burden you with that. But let it be said that there are four sets of considerations, ethical principles that need to be met. And I'm going to go through each of those from the little that I know about the basic science to date and the preclinical studies. What I'd like you to do over the next couple of days and in continued conversations is to think about what's the science, what's the clinical problem, and how do we bring those principles to bear.

The four are safety, the possibility of benefit, what the experimental design is going to be, and consent. Let's move through these one at a time.

In terms of safety, this is the crucial element. This alone, not so much that there is a consideration of a calculus between risks and benefits. They're not commensurable. You can't equate them. The calculus doesn't work. A risk/benefit calculus isn't meaningful in this first step. What's really critical here is that preclinical studies tell you that it is safe to proceed. The idea of doing something in a person for the first time, safety is paramount, not harming.

Here you could make an argument that there needs to be near unilateral consensus in the scientific community about safety. We've heard different issues

raised this morning about what those safety issues are. There are concerns about tumorigenicity. Where is this stuff going to go? Where is it going to grow? Will it not grow? Will I hurt the person in the process of putting it in? Will they be infected with something else? The preclinical studies should give you sufficient information about safety to say that it's okay to move forward.

If you can get assurance or consensus that there is safety, the next step would be the possibility of benefit. In an early phase experiment, it's critical that safety is met. It's desirable that there's benefit, but it is clear that the reasons why we do things in people for the first time is we're just not sure from our preclinical studies about whether this is safe in a human being. It's nice if you can get some benefit out of this.

Now, originally the paradigm that most ethics folks in the regulatory scheme work on is a little out of date for biologics. It was put into play for chemotherapeutic agents and the like in which phase I was dealing with toxicity and just checking for toxicity. That model doesn't seem exactly right, but it's the best we have to date. So, the question is, it's desirable to design an experiment that can show some benefit, but really what you're trying to get at -- and this is important in the scientific design, as well as getting consent and moving

forward -- that you can say that we want to get there, but truthfully this study is not about this. This study, the first time we do this, is not about curing you or the disease. It's about figuring out whether we don't hurt folks.

Here a thing called clinical equipoise is a sufficient metric. There doesn't really need to be consensus about whether there's going to be benefit. People are going to disagree. Is it 1 cell? Is it 2 cells? Is it the microenvironment? Is it this kind of cell or that kind cell? There is going to be disagreement, and science will give you the answer. As long as you're not harming people in the process, in some ways it doesn't matter quite as much. You want to get it right, but the history of science and medicine has shown us that our predictive abilities are not that great.

Here the word clinical equipoise is one used when you're in the middle. You don't know. It's a great word for Scrabble because it has a Q in it. So, you can use that. People will say that's an equipoise word. It doesn't mean that every scientist is going to be directly on that tight rope saying, yes, I'm 50/50 on this. I don't know. Every one of us, when we want to move forward in the clinical experiment, believes that this cell, this approach, this remedy is a home run. And we're wrong

statistically. If you don't think you're hitting a home run going into clinical experiment, you might not as well try. The numbers are just dismal about how well we do in this. But there should be some equipoise in the entire community of scientists about whether there's a possibility of benefit.

Now, design here is really crucial. It's not just about putting something into somebody, but the design itself has to be sound. There have to be ways to assess it, and there has to be fairness in the selection of the conditions and subjects. And I'll talk about each of these in turn.

For science to be sound, there obviously has to be some theoretical justification for it. You would like, with all of these preclinical studies, animal models and the like, to be able to explain, at least with the current theory or theories that you're working under, that the science makes some sense.

You also want to minimize risks and maximize benefits. Minimizing risk here means to use a safe product, and the question of the source becomes really critical here. At this stage of the science, from an outsider looking in, it would seem that it makes considerable sense to know exactly what that source material is. Dr. Rao mentioned some of this in his talk.

Those may not be the final set of criteria which you use to address this, but it's clear that some of that information is going to be vital at this stage of science.

What are the implications of that for donors? Well, obvious. Their privacy might be violated. People might want to go back and get more cells. We know that that problem is there. We just have to build appropriate fire walls and protections and mechanisms of protecting the privacy and confidentiality of donors. That's possible.

The key here is going to be to know whether the source is safe and then, again, another set of scientific considerations regarding what kind of tests we can do now. No matter what kind of tests we dream up now, within five years we're going to think of new tests that we can now do, that we want to do. Anticipate it now. We've done this over and over and over again where we've got a pot of stuff, whatever it is. If it's blood, if it's stem cells, if it's cord blood, it doesn't matter. We find a new test. We want to do it for everybody. So, we need to anticipate that in the donor recruitment and selection process. Again, I don't want to get diverted there, but again, you're going to want to think through those issues now if you're anticipating doing clinical trials in the future.

Linkage in testing, obvious results. The hazards here are not maintaining an appropriate fire wall

so that people's privacy and confidentiality aren't inadvertently violated. Coming up with sort of standard procedures that hematologists, blood bankers, and tissue bankers of all sorts are well accustomed to. These are not new issues about what the procedures are going to be for notification, what the notification procedures are going to be provided something goes wrong, provided you discover something. When will you warn? These are well rehearsed problems.

In terms of maximizing benefits, obviously you want to pick the best science. You want to try to hit the home run. This is obviously the intent of most, but that may mean some distinctions and differences between which product gets tested first in people. That's when the science gets a little interesting.

Outcome measures. This is critical in biologics. What's measurable and what's meaningful? How are you going to measure it? It's one thing to do this in a person and put it into brain, a black box. How are you going to get it? Are you going to be able to image it? Are you going to have to take a piece of it, a chunk of it, a chunk of it periodically, and what the implications will be about where you're putting certain cells and what they're doing and how you're going to model that. That's going to be crucial to figuring out whether this is the

right condition to start with. You might be able to do the same kind of learning somewhere else. The question is how are you going to get there. Is this going to be a meaningful result, not just something that changes over time?

Adverse events, another area that's received just a little bit of popular press and attention. Adverse events in biologics, gene therapies, vaccines, and the like. Again, we've got to know what we're measuring and we've got to figure out how we're going to report it, and when we're reporting it, where does that mass of information go and who's going to look at it?

IRBs are not equipped to look at adverse events. DSMBs may be equipped to look at adverse events, but may not communicate with IRBs. This is a problem across clinical trials. But as we're moving forward, especially with high profile trials, it would be important to lay out those mechanisms in advance about who is going to do what.

It may be that you construct your DSMB, if you have one, even though you might not construct a DSMB for a phase I equivalent sort of trial, but in a case like this, there might be good reasons for oversight, especially because it's sort of devoid of what once was the RAC mechanism and the like. And we can go into any of this in

detail. But you're going to need some folks who are pretty savvy with the science to help sort this out.

Fairness in selection is another criterion of doing good science. First, of the condition. In this country we have people who were effective at lobbying hard for their disease condition. AIDS and cancer are the most notable about being able to lobby political interests. It affects a lot of people. There were clamors for access to experimental designs. Those are legitimate claims. The question is early on what's the appropriate candidate disease to learn about the technology or the approach.

One criterion here may be what best advances a scientific goal. If one disorder requires the intervention of 20 things and another disorder might require the intervention of 1 thing, it's going to be a whole lot easier to figure out the 1-thing science than the 20-thing science. You can figure out which diseases and approaches you're thinking about, but the elegance of the science will probably help inform future efforts, even if that flies in the face of some pretty intense lobbying efforts. It's not to say that those diseases aren't devastating, but if you take this approach and there are bad outcomes and they're uninterpretable, we don't do well in the long run.

One other condition that's often advanced is there is a lack of available alternatives, and there's a

plus and a minus to this. I'm sick. There's nothing for my condition. Treat me first. It's a legitimate claim as well. It's a very powerful claim. The question is, though, from a position of consent, where you maximize the ability to sort of say there are no other alternatives, and so the risks and the benefits seem to not make a lot of sense, folks can't very well be positioned to give a meaningful, voluntary consent process. They're pretty vulnerable folks.

Among that, you're going to get questions of fairness in selection of subjects. Who gets to come in first? The stockholder, the first person in line, the first person who expressed an interest, someone who will donate to the lab, build a new building, has basketball seats of the major university? Whatever the major goal is, I think it's important to think how you're going to choose among subjects, and some of those criteria aren't just who got there first but a question about how that selection process is going to take place, and again, how you can learn the most from the science, especially early on.

The final part. No ethics person can get up here without talking about informed consent. It's our favorite and one of the reasons it's our favorite is because most scientists recognize that you have to do that, and so it's our way in. We know you're going to fill one

out. We know you're not going to like whatever the IRB says about the font, your language choice, and consent documents, but there are some other parts here.

Let me go through an informed consent, the standard approach, some challenges that are special to these sorts of things we're talking about, and avoiding something called the therapeutic misconception.

Standard consent. This is ethics 101. Informed consent is a process, not an event. Three major steps: threshold, information, and consent. Threshold means if you don't pass that threshold element, you're not allowed to play informed consent. It has to do with decision making capacities, sometimes termed competency. Competency is a legal determination. Most of us are not judges. Most of us don't want to be judges. So, we can make an assessment in the clinical or research world about decision making capacity, people having an ability to make a decision, take in new information, process it, use it to make a decision, and evidence that choice.

Critical components. Think about the diseases. Some folks for some of the neurologic diseases may have impaired decision making capacity or competency. Now, some of the ones bandied about today, not so much, but the question is going to be very important to think through what kind of capacity people have to make that decision.

Another threshold element is voluntariness. If you really have nothing else wrong with you, anything else to be considered in that situation, you may not be positioned to make a voluntary choice. I once gave an ethics talk and a woman came up to me. She raised her hand and she said, you know, I can never get people to sign the consent form. And I said, well, why not? I was thinking there must be some kind of strange science that they were going to put something in and it was like an X Files case or something. I didn't know what it was. She said, I can't get them to sign it. I said, why not? And she said, well, their arm is always tied to that arm board. I said, when do you recruit subjects? And she said, oh, in the pre-op holding area.

(Laughter.)

DR. SUGARMAN: Now, most of us are uncomfortable buck naked with 16 gauge IVs and our arms like this. It's definitely not a position of voluntary choice. You may volunteer to be there, but that's not what was intended by here. Some clinical circumstances position folks not to be able to make a voluntary choice, and we need to think through that.

Those may be pieces. You don't put those on your consent documents that the person has decision making capacity and that they're in position to make a voluntary

choice, but that's the spirit of the process of informed consent.

In considering and designing these, we should be talking about the consent process as a whole. Now, here this stuff is going to be more familiar.

Information. Right? Part of it is informed.

Disclosure. Disclosure has nothing to do with the example I gave you in the pre-op holding area. Disclosure has to be the kinds of information that the investigator gives to the potential subject. This stuff everyone is familiar with. Right? It's in the common rule. It's in the FDA regs. It's everywhere.

Procedures to be followed. This involves research, the risks, the benefits, the alternative, procedures to protect confidentiality, who to be notified in case you're a research subject, who to talk to if you're injured as a result of research, that you can opt out anytime. Those elements are clear. And, you know, they're pretty sound. It's hard to argue against any one of them, especially in new technologies. Just follow the rules. Check the boxes. Make sure they're all there and make sure you get them right.

Understanding here is really key. Now, understanding these elements. It's one thing to walk up to someone -- and I'll choose a non-neurologic disease -- and

research. A study is when the doctors and nurses look over your medical records. They study up on you. Who wouldn't sign up? So, the terms themselves are quite important.

And just so you know, the terms "clinical investigation" and "clinical trial" -- people don't have a clue what they mean. "Clinical investigation" is they wonder what went wrong. And "clinical trial" is like time for Wapner. There's no sense of what these terms are that we use when we bandy about.

Now, the other piece here that's going to be key with biologics is that the perception that biologics are natural and natural is better. Sort of like your poison ivy, right? Now, poison ivy causes problems, but when we understand natural or biologic, this is a difficult concept to communicate about the fact that this is something medical. It may be like a chemotherapy that there's some associated risk.

Get through that. You've got someone with decision making capacity who's been able to make a voluntary choice. You've given them information that you're required to give them in a way that's understandable to them. And then they make a decision and then they authorize their decision by signing one of those forms. That's basic consent.

The special challenges here I think are going

to relate to the source of stem cells. I would add -- I would be hard to convince not to believe this. Truman talked about the economist, you know, the one-handed and the two-handed. My friend Lance Dell at Davidson College talks about two-handed or evenhanded ethicist. The two-handed ethicist weighs all the alternatives on this side and all the alternatives on this side, and then washes his hands and lets you make you make your own decision. The evenhanded ethicist sort of says, there are some options on this side and there are options on this side. And you know? That one is just not right.

I tend to be evenhanded when I can be. Sometimes I can't know. I don't have a horoscope. I do use an 8 ball on occasion, but not for matters of real scientific importance.

But here the source of stem cells is going to need to be included in the informed consent process. The area that is charged in this area -- say it out loud, say it explicitly -- is the source of stem cells. If they come from embryos, it scares people, not all people, some people. Tell them. Some people will say, thank you very much, I don't want that. Other people will say, I don't care, it doesn't matter to me. But they need to know. Just include it. Figure out you're going to include it. These were derived we don't know. These were derived from

whatever you need to tell them.

There is a lot of uncertainty about risk, and I think that's going to be hard to communicate. It's always hard to communicate this and especially the first time you're going in. This isn't a novel, but the part that I mentioned about a biologic and what that means is going to be the trick here about communicating that kind of risk.

The other is the alternatives or lack of alternatives. It is no surprise that the list of candidate conditions is devastating. They're awful conditions with a lack of good alternatives in many cases. But in other cases, there are. If you're thinking about Parkinson's disease, there are pharmacologic interventions. There are fetal surgery -- I mean, we can debate and quibble about whether that's an effective alternative, but there are alternatives. And those alternatives need to be described in detail so people can make decisions because sometimes we've got something to offer. Those need to be incorporated even though there's going to be this move to move the science forward quickly.

Well, here the piece on biologics again plays into something that's common in clinical trials of all sorts. It's known as the therapeutic misconception. It's the belief that experimental procedures are directed primarily at therapy. There is a distinction. When a

doctor is taking care of a patient, the overriding goal in that case is the primary care of that patient. Something is not working. You shift gears. Right away it's about you, the patient. In a clinical trial, part of it's about you, the patient, the very critical part about it, but there's also another tension and that's of sticking to the science and finding out what happened.

There are additional procedures, even if both of those interests are aligned, that just aren't part of clinical practice. There's extra sampling to check for biologic effects for outcome measures. There are extra visits. That could be good or bad. There is going to be everything from questionnaires to urine and blood samples. Whatever it is you dream up, there's going to be something else.

When you talk to patient subjects on trials, they believe, even in phase I studies in oncology, which are about toxicity -- they can tell you the scientific rationale of the study, but they believe that they're going to get better as a result on this trial. Now, balancing candor and hope in these situations is very difficult. The challenge, though, is so that folks can make a decision to know what the stage of the science is and early on -- later on phase III, randomized phase IV, whatever -- the differences are hard to flesh out and you don't need to

quibble there. But early on it's real critical to know that therapeutic misconception exists.

Serving as a subject can be challenging for patients. We just don't. The phenomenology of illness tells us that we don't want to be treated as a subject. We'd rather not be treated like a patient, but it's better than being treated like a subject. We just don't like it.

An investigator's interests are, unfortunately, aligned with this. We want and need to personalize. We don't want to tell folks that this is about the science and not about them. It's tough. For those folks that are clinical investigators to walk into a room and say, this is about the science and not those things I wrote on my essay to get into medical school is a hard transition.

Moreover, it enhances enrollment. If people believe this stuff, this is a perennial issue in clinical trials. None of it is bad intentions, I don't believe, but it's very present and I think critically important in early phase trials.

Finally, these are additional concerns outside of the sort of box that we've been thinking through.

There are going to be some pressures to delay progress. I don't know why you decided to have this meeting out here in this outer galaxy of the beltway, but we're not downtown. We're not in the fray. There are

going to be pressures to delay. You say stem cells. People panic. There are going to be some pressures to delay.

But there are also going to be huge pressures to move forward. And the pressures to move forward are going to have to be balanced in these ways about the excitement and when it's appropriate to move forward. Again, it's just important to say these things. It's like the emperor's new clothes. It's just what it is.

So, in conclusion, the scientific and commercial interests, along with the hopes of patients, need to be considered in light of the scientific realities and not merely the aspirations. There is a clear set of ethical issues and considerations that we need to think through in moving to clinical trials, and I think it's critical to deliberate about these things explicitly as the process moves forward.

Thanks for your attention.

(Applause.)

DR. SALOMON: Thank you. That was really excellent.

So, to start the discussion, there were many points you made that I think are worthy of discussion. One that sort of picks up on a theme that we were discussing when we walked in you brought up under outcome measures. I

thought you put it very well by saying there was a contrast between what was measurable and what was meaningful. I'd like to hear some comments from the experts on that since outcome parameters in terms of these first clinical trials in these transplants is going to be obviously critical to everybody. That's not saying anything surprising.

So, there are people here at the table who have got their own disease stuff. Right? Some of you are doing Parkinson's disease, some Huntington's, ALS. So, could you guys maybe make some comments from your own area on what would be a measurable outcome parameter versus what would be a meaningful outcome parameter?

DR. FREEMAN: I think the first question is what's tried to be addressed scientifically in the trial, and if you've got a novel therapy where there are no alternatives and you have any reproducible, meaningful benefit, that would be important to ascertain in a phase I trial or even a controlled trial. But I think the hurdle gets higher and higher as you have more options and the science advances. So, I think it's really a moving target. Do you power a study based on a statistically significant change or a clinically relevant change or a change that addresses your scientific question is a separate issue based on where you are in the science and in the development of a new therapy.

DR. DRACHMAN: My favorite quote is Gertrude Stein who said, "For a difference to be a difference, it has to make a difference." I think that's probably relevant here. We've gone around and around the treatments for Alzheimer's and finally ended up with a CIBIC-plus, which means that someone other than a neuropsychologist can also tell there really is a difference, but it's very difficult.

DR. TROJANOWSKI: I think just to follow up in the area of Alzheimer's disease, it's still a moving target. I just came from the meeting of the WAC 2000, the World Alzheimer's Conference 2000, downtown within the beltway. It was announced yesterday in the Washington Post a vaccine that may eliminate plaques. That's really very compelling preclinical data. But patients don't come in complaining of a head full of plaques. They come in complaining of memory impairments. There's still, I think, controversy in the field as to whether eliminating plaques will convert these patients into those that die of a "tauopathy" because their tangles continue to accumulate or not.

There are going to be imaging agents shortly I believe that will image plaques, maybe tangles as well, but I don't think we have our arms around all of the measurable important and relevant things that one might want to

consider. Certainly the patients complain of memory, and that's the most important thing.

I think an innovation that's just developing that I see in the Alzheimer's centers, the 27 centers around the country, is interrogating the caregivers for their views as to what an outcome might be that is beneficial. Remember, these patients are cognitively impaired and cannot themselves always state whether they're feeling better reliably.

So, fortunately, the science is moving at such a pace that we hopefully will have all the measures in place when the vaccine -- they're actually going into people right now, but we won't know for certain right away.

DR GAGE: John brings up a really interesting point that be worth some further discussion about the ethics of it. So, the tauists versus the amyloidists or Baptists.

(Laughter.)

DR GAGE: We don't know really whether or not either one of those pathological manifestations are involved in the behavioral deficits that are seen, which are really what's important. But if you had a therapy which could eliminate the formation of the amyloid, even though you don't know whether or not elimination of the amyloid is going to change the nature of the disease, since

there isn't an animal model that is excellent for the disease, is that justifiable? You're asking a scientific question to some extent with the hope that there might be some therapeutic output. But is that something that -- obviously, they're going to do it, but where does that fit on our --

DR. SUGARMAN: No. This is the exact kind of conversation that ought to go on, and it's this conversation that often happens but in small groups and not among different groups of experts. This is what should happen with each trial as it goes forward.

Now, it happens sometimes behind closed doors, but because of really legitimate reasons for those conversations to take place behind closed doors. There are proprietary interests, but at the same time, they're very powerful in terms of what's going on in this trial and what's this trial about.

So, you have to say, what's this trial about? If it's to look at plaques and then say, does that correlation between plaque formation correlate at all? Do you want to know in that study even if that correlates with behavioral change? Or is that a separate study? Those could be discrete scientific questions involving different investigators and different outcome measures and how you power it. So, this is the precise kind of conversation is