We're going to discuss today the definition that you two gave which is making a genetically identical copy in terms of organisms. And I want to begin by distinguishing with you the terms reproductive and therapeutic cloning. Reproductive cloning refers to making a whole organism, doing cloning with the intent, and here we are in our board of life, same place we were last time, stem cells cloning, let's not dwell there. Reproductive cloning, the stuff that covers' of Time Magazine are made of. The outcomes of reproductive cloning, the desired outcome is that you get the whole organism. And I want to distinguish that from therapeutic cloning where the desired outcome is that you get some kind of cells and specifically stem cells. So reproductive cloning is the stuff of movies, it's the stuff of science fiction and of people having fun with covers of the New York Times Magazine. It's also the subject of scams. And I'll tell you at the outset, there are no human clones now. Human cloning has not yet worked, but I am sure someone somewhere is trying. And we'll come back to that later one in the lecture. The other type of cloning, therapeutic cloning is something that extends directly from our lecture last time. When you think about stem cells and you think about using those in repair, one of the issues that comes up, and this comes up in any kind of repair mechanism, repair therapy, transplantation of any kind is the question of autology or self or matching, whether or not the donated tissue genetically matches your own If it doesn't your immune system is going to try to reject it, and if it does you have a lot of problems. The sense of therapeutic cloning is that one may be able to take cells from an adult person and turn them into an embryo and then use the embryo cells, I'll tell you how in a moment, and then use the resulting embryo and the inner cell mass of the embryo to make various stem cell lines, as we discussed in last lecture. And the promise of therapeutic cloning is this autologous nature to it, that you would get something that was an exact tissue match. So let's discuss a bit more, why one might want to do this various types of cloning. In reproductive cloning there are some senses of replacing people who have been lost, some way of improving or overcoming infertility. Spare parts, this is the stuff that some good books have been made of. House of the Scorpion from Nancy Farmer. If any of you have read that it's directly relevant to this. If you haven't, House of the Scorpion it's called. It's a very interesting book that thinks about a time in our society where we can clone exact copies of ourselves. And these copies are made brain damages. And they are kept in hospitals and they are used for spare parts as the real person ages. OK? Sounds weird, sounds peculiar, sounds unethical, but it is possible. And I suspect there are people who would be quite keen on having this as something that could be done. Useful producer animals, there's been a sense in the agricultural industry that if one can make identical copies of particular animals it would be very useful in getting whole herds of goats, for example, that are making very high quantities of a protein that is pharmaceutically useful, some kind of drug for example. And there are in different ways. That kind of approach has already taken. Therapeutic cloning, as I said, autologous stem cells and the use, which I may or may not have time to touch on, of these cells in correcting genes which are mutant and which are bad. But let's step back for a moment because the question of cloning has a long history. And its history really started with a question that we have dwelt on for a number of lectures, and this is the question of how cell types are determined. And the question of when a cell type is determined, whether or not that is accompanied by some irrevocable change in the DNA. So this cell type determination, or let's say differentiation because that's the end point, as you know, is cell type differentiation caused by DNA change? Or let me actually say DNA change or DNA loss, which is the big one. And this question, and you could imagine, as you form a retinal cell or as you form a skin cell, you might get these different cell types because you throw out, you actually delete from the genome whole batteries of genes. OK? Or you make lots of copies of another gene. So you may actually change the DNA. You kind of know the answer to this question because I've already told you that all cells have pretty much got the same amount of DNA, the same type of DNA. And what's important is whether or not different genes are active or not. But how did I tell you that? How was I able to tell you that? So let's spend a few minutes exploring that. So the first question that was asked in terms of the cell type differentiation question was the question of cellular potency. Where cellular potency, and we're on number two of your handout, where cellular potency asked in an early embryo, when can an early embryonic cell or set of cells support growth of the entire organism? In other words, at what time during development is there totipotency, is there complete information in the cell? And this is to exemplify the kind of experiment that was done, as exemplified here in the sea urchin where you can take an eight cell stage sea urchin embryo and transect it in one plane or another plane. And if you do it in one plane you get an abnormal embryo and a normal embryo. They say abnormal but it's sort of normal-ish. And in the other plane you get out two normal, but small, larvae. OK? And what this said was that the cells of this eight cell embryo are not equivalent to one another. And, indeed, even four cells put together, if you bisect in the wrong plane, was not sufficient to confer totipotency to those cells. So at the eight cell stage in the sea urchin, the cells are not totipotent. And you can do similar types of experiments in many organisms. And what you find is that in the very early embryo, you can often split the embryo into its component cells and you can get out a number of genetically identical organisms. This clearly happens in humans in the case of identical twins. It happens in armadillos routinely where armadillos, for their own reasons, split into eight cells at the eight cell stage, and you get identical octuplets out of most armadillo pregnancies. OK? So there's totipotency but it's transient. And this transient nature of this cellular potency, so the embryonic potency is transient. And the question was then raised why? Was it something that happened to the cytoplasm or was it something that happened to the nucleus? And this is where we get into the questions of nuclear potency and the beginnings of cloning as we know it. And the technique that you need to know is called somatic cell nuclear transfer. Abbreviated SCNT. All right. So this is what SCNT looks like. This is number three on your handouts. And it goes like this. One takes an egg, early embryonic cell that you know is able to support total embryonic development with its own nucleus, and you go in and you remove the egg chromosomes. You can do this because most eggs are arrested at meiotic metaphase two. The chromosomes are condensed and they are gelatinous, and you can actually remove them. I'll show you a movie of this in a moment. You can remove these egg chromosomes, and you can then get a nucleus from

some adult or other cell type. And you can put it in a glass pipette, and you can pipette it into the enucleated egg. The egg heals up. It's now got a substituted nucleus. And you can go and you can ask what this egg which its somatic nucleus can do. And the somatic cell nucleus is diploid so you don't need fertilization because you've already got the right complement of chromosomes. And if you do that, as was done in frogs in the early 1960s, you get results like this. The host egg came from a frog which was brown and the donor nucleus came from a frog, or cells of a frog which was albino. And that was important because when you looked at all the cloned frogs they were all albinos. OK. And so you could see that they came from the donor nuclei. Great question. See me afterwards. I actually don't know the answer. That's a great question. OK. Here are the cloned frogs. OK? And you can get buckets full of these things, but. I've seen buckets full of these things. OK. But. OK. But before the but. So let's do a couple of things here. So want I can tell you is that in a 500 cell frog embryo, one can very clearly distinguish cellular potency and nuclear potency differences. So if you take any cells from a 500 cell frog embryo, you isolate the single cells and culture them as single cells, none of them will form an embryo. However, if you isolate nuclei from each of those cells and transplant those nuclei each into an enucleated egg, 100% of them will form normal embryos. So there is a massive difference between cellular and nuclear potency at that stage. It tells you that the nuclei are totipotent and the problem was with the cytoplasm of the cell and that the egg cytoplasm has got something in it that is special to support development. So that was a very positive result, OK, that you could get these cloned embryos, these cloned frogs from nuclei that by cellular experiments were not totipotent anymore, but -- But, and this is really important, if you did that experiment with nuclei derived from older and older embryo, here's blastula, gastrula, neurula tail bud and so on all the way up into adult cells, the frequency of embryos that arose from, or frogs that arose from the somatic cell nuclear transfer plummeted. So if you did the experiment at blastula stages you got 80%, even 100% success rate with the cloning with your somatic cell nuclear transfer. If you did it at gastrula it would drop to 50%. By late gastrula, which is just in frogs, about five hours after late blastula, you were down at around 15%. And by the time a day later had gone by and you were a tail bud embryo, you were close to zero percent success. So although nuclei were totipotent at some stage, there was a tremendous decrease in the potency of the nuclei as development went on. And so that raised issues, which we'll come back to in a little bit. Now, after the success with frogs, people started looking to mammals immediately to see if they could clone mammals. And there is a real checkered history here and some shady science and some claims particularly that in mice one could clone mouse embryos and could do it with high efficiency. And it turned out that all of that data was faked, and that really put a damper on the field for several decades. And it wasn't until the folks in Edinburgh in 1997 tried again and did it with an open mind that they were able to clone this sheep, Dolly, who never knew how famous she was, who was the first mammal who was cloned by nuclear transfer. So how does nuclear transfer work in mammalian embryos? The principle is identical to what I've shown you in the frogs, but I'm going to show you a few movies to give you a sense of how it's really done. So this is a microscope that's set up for somatic cell nuclear transfer. And these things here are micromanipulators, including injection and suction devices that I'll show you in a minute. I'm going to show you a series of three movies that were made by a very talent MIT graduate student, Kevin Eggan who is now a fellow over at Harvard, in Professor Rudolf Jaenisch's lab, one of my colleagues over at the Whitehead Institute. And the first movie is going to be the isolation of nuclei from somatic cells. So here is a glass pipette, and Kevin is drawing up a cell into the pipette. And he is going to spit out, he has, you'll see it again. He has spit out the contents of the cell other than the nucleus. So there's this little thing in there that's a nucleus. So here's another cell. He's pipetting it up and down to break it open and is retaining the nucleus because it fits well in the pipette and doesn't come out as readily as the other cell contents. There goes the cell into the pipette, and here comes the cell debris, and in the pipette is retained the nucleus. OK. Step two, removing the chromosomes from an unfertilized egg. This thing is a suction device that sucks and holds the mouse egg. Here is a microinjection needle. And it's not pointed because it's actually being helped by an electric pulse to enter the egg. And Kevin is now pulling out the metaphase plate from an egg. Those are the chromosomes from the egg. How does he know where they are? Well, he's a smart guy. And if you've looked at enough mouse eggs, you can actually see them under the right microscopy conditions. OK. So there goes the pipette again and here comes some more chromosomes. And there you go. OK. Easy when you know how. There. OK. So once you've got your bucket load of eggs that are enucleated and you've got your pipette full of somatic cell nuclei, you can put the two together. So here's your enucleated egg held on by suction and here's your pipette that has got those nuclei that came from the somatic cells. Watch the barrel of the pipette or the microinjection pipette and you will see it. There is goes. There's a nucleus going down into the cell. And if you watch carefully we will see when the pipette comes out. You really have got to get it out of there. That nucleus is gone. OK. Here's another one. There's the nucleus. You've got to watch. There it goes. You can actually see it going it out if you look carefully. He's really making sure he pushes it out. And there, another nucleus somatically transferred. OK. And the third one here is the nucleus. A little pulse of electric current to get the pipette in, and then you push the nucleus out by pressure, and there you are. And the last one, here's a lost nucleus. There is goes. All right. OK. So this works. It does work. These mice are living proof that somatic cell nuclear transfer gives clones and so is Dolly, but there are issues. There are many, many problems. Cloning by somatic cell nuclear transfer is very inefficient. It depends somewhat on the species one has looked at, but it is generally around or less than 1%. So fewer than one in a hundred of those mouse eggs actually make it on to give you a mouse. The ones that do, although they might look normal, turn out all to have something wrong with them. So in mice 100% of the animals that you get out have got something wrong with them. And this is probably true in all clones that have been made. It was true of Dolly who died an early death due to arthritis and various other issues. And it's true in essentially every animal, in all animals that have been carefully examined. So what are the problems? This is to remind you again that cloning efficiency from adult nuclei is very low. One of the problems, and these animals would not be viable, is something called large offspring syndrome. This is a normal newborn mouse and this is a cloned mouse. And you can see that it's at least double the size of the normal mouse. This is

mouse and this is a cloned mouse. And you can see that it's at least double the size of the normal mouse. This is the placenta from the normal mouse and from the cloned mouse also enormous. So there is an issue here with the size of embryos or the size of the animals. There's also an issue with lifespan. This is a survival curve for mice. And mice cloned animals are pretty much all dead by about 750 days after birth. Whereas, for most wild type or even animals made by artificial reproductive technologies, your survival is way, way longer than at. At least double that time. So there is an issue with survival in general. And I'll show you a little bit, I pulled out a couple of panels of some primary research data to show you that the reason these animals are abnormal is because their gene expression is abnormal. OK? So this is a PCR-based assay looking at various RNAs from various different genes. OK? These are, each of these names up here is a gene name. It doesn't matter what they are. And each of these white bands here is the demonstration that an RNA is present. In a normal embryo there is a band under each of these gene names indicating that the RNA is present. In a cloned embryo, and this is true for all cloned embryos, you see that at least three bands are missing. And here's one I didn't diagram, didn't put an arrow on. Four bands are missing. And in different cloned embryos different RNAs are missing. So you can show that in cloned embryos where you look gene expression is abnormal. Why is gene expression abnormal? Well, there are a couple of reasons. One is that in fact we're wrong and that the DNA content of cells really is changing as they get older. But that's not supported by projects like the Human Genome Project which suggests that the genes, in fact, are stable in all cells. A much more plausible and accepted explanation of the data has to do with the control of gene expression. And an aspect of the control of gene expression that we have mentioned, although not by this particular term. And so I want to introduce you to a new term which is epigenetics. Epigenetics means outside genetics. And this refers to the control of gene expression that does not directly bear on the DNA sequence. We can have an argument about this later, but that's how I'm going to define it now. This refers to the regulation of gene expression -- -- that is not base sequence directed. That is not directed by the DNA base sequence. All right. And I wrote it for you. Let's look at some examples. This is a cloned cat. This is a kitten and this is its mom. And you can see they're very cute but they are different from one another. OK? They're different but they've got exactly the same DNA content. They're different. They're expressing different genes. OK? This is where we've been. Let's move somewhere else. I would think we might have someone who is one of an identical twin pair in the class. Anyone an identical twin? Oh, yeah. Well, we're statistically borderline. They're one in a thousand. So we don't have it. Do we have fraternal twins? Are you a fraternal twin? One. OK. All right. A few. OK. So there we're OK. All right. OK. Identical twins. Identical twins are generally not absolutely identical. They might look a bit different, they might have different behaviors, and they may have different more quantitative traits. The term concordance refers to whether or not twins share a trait. And that's particularly useful in looking to see whether diseases are causes by DNA based mutations. There's something called scleroderma which is a skin disorder that has 98% concordance. If one twin has it the other twin has it. And it is directed by specific changes in the DNA sequence. On the other hand, asthma only has a 54% concordance. And that suggests that asthma is directed, this is a high concordance, it's higher than fraternal twins have, but it certainly doesn't reach the heights of scleroderma. And what the data suggests is that scleroderma is caused only due to changes in DNA base sequence and asthma is caused due to changes in the DNA base sequence as well as something else, and the something else is believed to be changes in gene expression that are regulated above the level, at a different level than the base sequence per se. Now, if we think back to molecular biology and the regulation of transcription, I pulled this slide from one of your previous handouts, your gene, your promoter and your promoter activity leading to production of RNA. That is the level of gene expression that seems to go wrong in cloned embryos. We talked about regulation of transcription by binding of transcription factors to the promoter. And you've just had an exam on this previously. And also by something called chromatin modification. And epigenetics has to do with chromatin. Again, this is an old slide. You've had it previously. And I pointed out to you that DNA is packaged into tightly wound coils called chromatin by winding around particular proteins. And the particular proteins it winds around specifically are called histones, and these histones and the DNA wound around them are inhibitory to transcription and they must be loosened up or removed to allow transcription to take place. So bear that in mind while I tell you something else that can happen to DNA. DNA can be covalently modified, and this is on your handout. This is number six on your handout. DNA can be covalently modified. In particular, cytosine, the base cytosine can have a methyl group added to its 5 position, and it is now called 5-methylcytosine. And this does not change the base sequence of the DNA, but it does have a profound affect on whether or not the DNA is transcriptionally active. So let me go through a cartoon with you to indicate how this is, and then I'll come back to the relevance of this for cloned animals and their abnormalities. So this is number seven on your handout. The DNA that I've shown in black, and I've shown double-stranded DNA as a single line here so don't get confused, is wound around purple barrels of histone proteins. And this chromatin configuration where the DNA is wound around histone proteins has to be removed or has to be modified to activate transcription. When the histones are removed the transcription factors can do their thing and you can get transcription taking place. And it turns out that when the DNA is methylated that does not happen. Methylation prevents the removal of the histones from the DNA template, from the gene, and thereby prevents transcription from taking place from a gene. OK? So on the handout that I've posted on the Web, I have put an explanation of why methylation prevents histone removal. I'm not going to go through it in class, but you can go and look at it for your interest on the Web posted. OK? But what I want you to have in your minds now is that methylation prevents DNA removal. Now, methylation therefore, because it can repress transcription, is used by this body to use by the cell as a mechanism to regulate which genes are active and which cell type. So in cell type one where there's a gene that I have depicted as so, double-stranded and there's a promoter, no methylation, it's expressed. That same gene in cell type two might be methylated and therefore not expressed. This is number nine on your handout. Reciprocally gene two might be methylated in cell type one where it's not expressed, not methylated in cell type two where it is expressed and so on. And what you can get from this is that in a particular cell type a set of genes will have a characteristic methylation pattern. And you can get a kind of methylation profile of the entire genome that dictates which genes are going to be active and

you can get a kind of methylation profile of the entire genome that dictates which genes are going to be active and which are not active, or is one level of regulation that dictates which genes are active and expressed and which are not. There is another level of methylation that has to do with what's in the egg and the sperm and what methylation patterns are there. And it turns out that not only are there cell type specific methylation differences but there are gender specific differences. And the methylation patterns of female genes, of genes in the female are different than some of the methylation patterns of genes in the male. This particular kind of methylation difference is called imprinting. Here's an example. Here's a gene. Gene one in the egg has a particular methylation pattern. That same gene two, gene one in the sperm has a different methylation pattern. OK? And this difference makes it a so-called imprinted gene. Here's gene two. Also differences between the male and the female copies of the genes. And here's gene three which has the same methylation pattern in both egg and sperm and is therefore not imprinted. So there are these two kinds of methylation that take place, the cell type specific and the gender specific. And these all get put together into a complicated arrangement of changing methylation patterns during development. And I'm going to go through this. This is number ten on your handout. And I'm going to go through this as an animated cartoon. So here is, represented by the colors, the methylation pattern in the egg and in the sperm. They are imprinted with their own methylation patterns. During fertilization they join to form a zygote which goes on to make an embryo. Now, early on during embryogenesis some cells are set aside. And we looked at this with determinants in Caenorhabditis. Some cells are set aside to make the future germ cells. And very interestingly as that happens there is a demethylation of all the imprinting of methyl groups. And as those germ cells then go onto become egg or sperm new methylation takes place and you get the imprinted patterns put back on egg and sperm appropriately. It's a complicated process. And if you think about it a little you'll see there are some real issues there, but I'm throwing this out as a cartoon. Also in the embryo new methylation patterns arise that I've called embryonic methylation patterns. So there are enzymes that are adding these methyl groups onto cytosine, and there are a bunch of different enzymes. And in the embryo there are new methylation patterns that arise. And as the embryo goes through its thing and makes its different cell types, additional new methylation patterns arise. I've called these somatic methylation patterns. And they are different in the different cell types, as I've just shown you. So there is this complicated way of changing methylation patterns during development, and this has implications for the chromatin structure and it regulates the expression of genes in different cell types. It is one important level of gene regulation. And for cloning purposes you should know that adult methylation patterns never normally revert to embryonic patterns. The germ cells are set aside very early during embryogenesis, and once that has happened you go on and make all these new methylation patterns and you never turn them back. OK. So what does this have to do with cloning? Well, the problem with clones and the problem with gene expression in clones, it is believed, is that there is a problem with changing the methylation patterns and the chromatin structure back to the embryonic structure. So in normal developments, I've summarized the cartoon that I just showed you in complicated form, you move from some kind of egg-sperm methylation state that moves on, that allows you to move on to activate the early embryonic genes. And because you have put in the appropriate embryonic methylation patterns. And then in a stepwise way you go on to act two, form the adult methylation patterns and activate the adult genes. OK? So there's a connection between the methylation patterns and correct gene expressions. This is a summary of the big cartoon I just showed you. After somatic cell nuclear transfer there are three possible outcomes with regard to methylation patterns and with the prognosis for the health of the resulting embryo. One outcome is that this adult donor nucleus that has its adult methylation patterns, when injected into enucleated egg, will undergo some kind of reprogramming and will land up with a normal embryonic set of methylation patterns. If it does that it will be able to support normal development. However, it is believed that this very, very rarely, probably never happens. What is much more likely is that this adult donor nucleus, when injected into an enucleated egg, goes on and is partially reprogrammed by the enzymes that are present in the egg. And it goes on to set up some kind of abnormal set of methylation patterns, some of which look like the embryonic patterns, some of which look like the adult patterns. And it goes on correspondeningly to activate gene expression abnormally. And, therefore, to make an abnormal embryo. And then a third outcome is that you just don't reprogram this nucleus at all, and it's an adult nucleus sitting in the egg. And in that case you get no embryo resulting from the somatic cell nuclear transfer at all. So it is, and this is number 12 on your slide, on your handout. So the notion now is that abnormal clones are abnormal because of an inability to reprogram the DNA, they methylation patterns and the chromatin structure to turn you into a normal embryo. So let's move on to the final thing I want to discuss with you today which are issues. Issues with somatic cell nuclear transfer and the whole notion of cloning. And I have a list of them here. They fall into two categories. One are the ethical issues and the other are the methodological issues. We raised last time the ethics of using human embryos to make human stem cell lines. And this ethical issue is still there. Is it acceptable to take a human embryo at the blastocystic stage, when it's a ball of cells and to harvest it, to kill it, to take the cells and to make stem cell lines? Someone asked me whether you couldn't take one cell out of that embryo and make a cell line out of that embryo and let the rest of the embryo grow. And in theory you actually could do that. It would be a pain in the neck, but you actually in theory could do that. OK? But that's not a possibility that's really being seriously entertained. This whole use of human embryos to support actually making a whole organism has its real issues. What if you do that and you land up with as you do in the mice and the cows and the sheep and the everything else? You land up with a whole bunch of abnormal human beings, what are you going to do about this? What's the fate of them, what's your commitment to these abnormal babies and what is the commitment of society here? These are very big issues. Another issue, which is a very real one, is where are the eggs that you're going to do all this somatic cell nuclear transfer going to come from? So there was a recent report from a Korean group who had actually made a human stem cell line after somatic cell nuclear transfer. They had used 650 human eggs. And they had used 16 women to donate these 650 eggs. At the present rate of making clones and making stem cell lines, you would probably need at least 500 to 1,000 human eggs per attempt to get autologous stem cells. Now, I told you previously, when we talked about assisted reproductive technology and you retrieve oocytes

from the ovary and do in vitro fertilization, you usually get about 20 oocytes every time you try to get eggs from a donor. That's a lot of people who have to donate these eggs. And if you're actually going to try and grow these into babies that's a lot of surrogate mothers. So this is an issue that has not been resolved or even discussed really very well. But these are the ethical issues that make these scientific issues move into the realm of legislature. There is currently a federal ban on using federal money to make human stem cell lines. So you cannot use money from the National Institutes of Health to do this. There is no actual ban on trying to clone humans per se. There is a gridlock because it's not clear whether to separate therapeutic and reproductive cloning from one another, but these are issues that are very loaded. And, as I mentioned last time, in the Massachusetts House we just approved the use of private funding to make human stem cell lines in Massachusetts, but not the use of federal funding. We cannot do that. OK. Methods. To date no primate has been cloned. There is something difficult about cloning primates. No human, no monkey has been cloned yet. Why? What's the problem? Not clear at this point, but I suspect those problems will be overcome in the near future. OK. Some other thoughts. What about these eggs? I told you the source of eggs for somatic cell nuclear transfer in humans is a big issue. What if you could turn ES cells into eggs? Could you treat then with something and turn them into eggs? And that would overcome the need for donors. Well, in fact, an experiment was published a little while ago showing that you could take ES cells and you could activate them in various ways. And activate expression of these proteins that you should recognize, ZP1, ZP2 and ZP3. Remember those? Yes? OK, the zona pellucida proteins. So that you could take these cells and at least start turning them into eggs. That's the sense that you might be able to do. OK. And then finally the question of how can you reprogram these adult nuclei to make the whole process more efficient? And I'll end with telling you two thoughts that are being entertained by people in the field. One of them is to take an adult donor nucleus and to try to reprogram it into an embryonic state sequentially. So if you take the adult donor nucleus -- Thank you. If you take the adult donor nucleus, you inject it into an enucleated egg, you let it partially reprogram, and then you take the nucleus out of that partial embryo and put it into a new egg, that nucleus does much, much better. And that's true in frogs as well. And you can then sequentially reactivate the genetic program. And that's actually one of the best arguments that the genetic material really does stay the same. And it's a problem with the chromatin structure of the DNA. And the other dream that people are pursuing is to take these adult donor nuclei and incubate them in some kind of reprogramming extract, some mix of proteins that will magically change the chromatin structure of the nucleus and allow it to revert to a normal embryonic nucleus so it an go on and do its thing. And I am going to stop there. And thank you very much.