

RD: Our next speaker is Philip Damiani, and I had the pleasure of meeting Philip yesterday at a luncheon. And he looked really, really scared, up on the podium. And I think part of his looking scared comes from some of the criticisms that we've heard of cloning, and its use as a tool in conservation. And I think Philip would be one of the first people to admit that this technique isn't a panacea. And I was quite impressed with the way that he dealt with this notion. But I do think that this technique has some very interesting ramifications for how we look at conservation biology, and how we look at, perhaps, captive populations.

Philip is at The Advanced Cell Technology Cloning Laboratory. He's the lead scientist or has been the lead scientist on the Gower Cloning Project there. And it's my pleasure to introduce him for his talk, entitled Recent Advances in Cloning and its Application in Conservation Biology. Philip....

### RECENT ADVANCES IN CLONING AND ITS APPLICATION IN CONSERVATION BIOLOGY

Philip Damiani, *Research Scientist,  
Advanced Cell Technology*

PD: ... Today I'm going to be talking about most of the research that was done in our laboratory which involved using what we call interspecies nuclear transfer, or interspecies cloning technology, to do some work on a species called *Bos gaurus*, or known as the gaur.

To start with, I usually start off with a definition of what's a clone. Most people think of clones as identical copies of an individual who donated their DNA. And that's correct. In the sense that when we use cloning technology, we are trying to replicate an individual who is either deceased or is currently living.

But cloning technology, itself, is not new. Mother Nature's been doing it for a long period of time. We don't clone dolphins, and we have no plans on cloning dolphins, either. (Laughter) Though I think, actually, in China there supposedly is a project where they're trying to clone the river dolphin.... (Pause)

But, as I was saying.... For cloning technology, we do try to replicate individuals, but Mother Nature's been doing it for a long period of time. Particularly when you're dealing with identical twins, or even with parthenogenetic species. There's also two types of cloning technology that I'm going to give a brief overview.

One is what we call embryonic cloning. And then there's the newest technology, which everyone's reading about in the newspaper because of Dolly, is what we call somatic cell cloning. And a somatic cell for people that are not familiar with the term is any cell in the body that's not the gamete. So we can take skin cells we can take fibroblast cells, epithelial cells and, technically, clone from that technology.

With embryonic cloning what we do is, we take an embryo at a particular stage of development. And, usually, it's the stage of development in which the embryo is still totipotent. Which means the embryo itself is made of individual cells called blastomeres. And we can take those blastomeres usually before this stage of compaction in embryonic development and separate them out into individual components.

Those individual blastomeres are actually what we call totipotent meaning that they can give rise to a whole new individual. And a

common way of doing this or a common process in nature is identical twins. So identical twins are formed from one embryo, usually at a two-cell stage, in which, for some reason, the *zona pellucida* which is the protective coating of the embryo is destroyed or damaged. And both blastomeres separate out, and we have two identical offspring.

In cloning technology, we've actually been doing embryonic cloning for a long period of time. There was a lot of research that was done in the early '80s, and a lot of companies that originated from that technology. And what they do there is, they take, usually, about an eight-cell, or a 16-cell, embryo if we're looking at the bovine species and separate them out into individual components. And, at that time, we would then take another egg, from another cow remove the DNA from that egg. Take the blastomere and fuse, using an electrical pulse, the blastomere into that enucleated egg.

The limiting factor with embryonic cloning is that we are only able to make the number of identical clones that have the number of blastomeres present. So, for instance, if we took an eight-cell embryo, we'd only be able to make an eight-cell . . . We would only be able to make, technically, eight identical clones.

With the invention of somatic-cell cloning, we utilize a skin sample. And, for our company, we are using a fibroblast cell line. We can take a tissue biopsy in the laboratory; dissociate that up into individual cells; produce a cell line.... (Pause) We can take an individual. . . (Laughter) I usually put that up, because most people think that's what we do in agricultural cloning is, we make identical copies of elite cows. And that is true but that's not necessarily the case for conservation.

These are just some of the species right now that are cloned to date. And, as you can see, the list is getting longer and longer. Originally, we started off in embryonic cloning just with bovines, mice, and then sheep and goats and, recently, the monkey. And I think that's sent a shiver through the community. Because it came out that . . . That report came out at the same time that Dolly was produced or after Dolly was produced and everyone thought that meant that human cloning is right around the corner.

With that monkey being produced, Tetra she was actually produced by embryonic cloning. So it's not a new technology in the sense of and it doesn't incorporate what we call somatic-cell cloning. To date, now, with somatic-cell cloning, we've been able to produce bovines, mice, rats, sheep, goats and recently the pig, which is, in my field, a huge feat.

This is what I was talking about embryonic cloning. You see the embryo at the that would be about an eight-cell embryo or so. We divide those up into individual blastomeres. And then these are the numbers maximum number of clones that can be produced.

With somatic-cell clonings we take a tissue sample; produce a cell line into the laboratory which could be fibroblast, epithelial cells it really doesn't matter right now. You know, neurons, of course, we can't use, because they don't divide. But we can then take the individual cells, enucleate the oocytes. And enucleation means taking out the nucleus, or the DNA, from the egg, and then fusing those. And the maximum number of clones is technically infinite. So if we have 100 cells that we're working with that day, we technically can make 100 clones. But you'll see in a minute that that's not necessarily always the case.

So what's required to clone? The main part of the cloning procedure is oocytes, or eggs, and we need a source of these oocytes. In the bovine field it's very easy to get these eggs. Most of us eat meat, so we go to a local slaughterhouse and pick up ovaries. We pay for them. They're very cheap. Otherwise, they go into dog food.

We then take the ovaries back to the lab. We have a team of people that's all they do all day long is actually just aspirate eggs out of ovaries. And then we have an oocyte maturation system, which matures the eggs to become metaphase 2 that's the stage of meiosis that we use the eggs. And it's important that we can only use functionally mature eggs, because those are the only eggs that are developmentally competent.

We then, of course, need a source of donor cells. Those donor cells can either be embryos, or fibroblasts, or any cell line. Then there's the nuclear transfer, or the cloning procedure, and each lab does their procedure differently. There's an egg-activation method. This is extremely important, because the egg is normally sitting at an arrested stage of meiosis. And it's waiting for a sperm to come along and to penetrate. And the signal that the sperm brings along is what allows the resumption of meiosis to occur. So even though we can fuse a new cell in, which would have new DNA, the egg is still going to be sitting there, waiting for the sperm to come along. So, with oocyte activation, we mimic the fertilization step. And, again each laboratory does this differently.

We of course need an embryo-culture system. Right now, in the bovine, we transfer what we call blastocyst stage of embryo, which is about day seven of development. So we have to culture the embryos to mimic the oviduct or, at least, the oviduct environment. Depending on what species you're working on, it could be as short as one day or as long as seven days. And then, of course, you need embryo-transfer techniques. We need to get the embryos into the recipients.

This is just kind of an overview of nuclear transfer and I'm going to take apart some of these steps. I had a video that actually shows the procedure. It won't play on this projector. But if anyone wants to see it, I'll load it up and I'll have my laptop out front ... or after, during the break, or so.

But the oocyte enucleation process is the stage that removes the chromosomes from the oocyte. And to visualize the chromosomes at least in the bovine species we have to use a DNA-specific stain. In other species it's not necessary, because the oocyte is not as lipid-rich, or as dark. Once the egg is enucleated, we're left with what we like to call just a bag of cytoplasm, and all the nuclear material is removed. And the egg then becomes the recipient for the donor.

The egg, itself that so-called bag of cytoplasm is what's actually going to allow future development, or embryonic development. And it's very important that, like I said before, that we have a fully matured egg. It doesn't necessarily mean that even though its nuclear DNA is fully mature, the cytoplasmic maturation may be abnormal, and we don't get any development.

This is the enucleation process. What we do is, we have the chromosomes right here. We have an enucleator pipette. We can go in and just, with a vacuum pressure this is all done with very fine glass tools. We pull the DNA out. We're finding out now that our future what we call cloners, are students that, growing up, were very good with video games. Because this technology requires very good eye-hand coordination which, you can see now, I don't have, because I constantly use my hands when I talk.

But the people sit at the scope, and they, basically, are looking through the microscope and they're doing all the manipulations with joysticks. So all the Nintendo and PlayStation kids are now becoming, possibly, future cloners. (Laughter) And I'm serious about that. Because we had a new person start, and it only took her two months to learn the whole cloning procedure. And when I was trained, in grad school, it took me at least six solid months to become very good at doing that. I was a geek I was reading I wasn't playing video games when I was growing up.

The nuclear-transfer procedure, itself, requires a transfer of the donor egg, which is a diploid set of chromosomes, into the oocyte. What we do is, we pick up a donor cell, again, with a similar pipette; insert it back through the *zona pellucida* of the egg; and then just we don't actually inject it into the egg in our laboratory. We actually place it alongside the cytoplasm of the egg. And then we use an electrical pulse, that fuses the egg, and fuses the cell into the egg.

And what that does the electrical pulse is rearranged. The cytoplasm or the lipid bilayer of the cells forms pores. The cell gets incorporated. Not only the cell, but the cell contents their cytoplasm and the DNA, of course. And then we activate the egg and culture it for a long period of time. And this shows the transfer, itself. So we're putting the cell and it sits on the outside. We apply the electrical pulse, like I said, and then the whole cell becomes incorporated in there.

So, to summarize.... We establish a cell line in the laboratory; recover the oocytes; mature them; enucleate the oocytes; and then do the actual procedure of transferring the donor cells; fuse cells, activate and then culture. And then, eventually, transfer, if we have enough embryos to produce.

Now I'm going to talk about some of the roles that cloning could potentially have in conservation. But some of the major issues that occur with cloning is: Why produce identical animals? And this particularly comes up when we combine the words cloning and conservation. The main issue is that cloning technically can decrease genetic diversity, because we're producing identical clones of an individual. I agree. Preserve habitat instead of cloning, because cloning is expensive.

And I'm not going to lie. I can tell you right now that cloning, right now, is expensive. The main reason and I'll discuss this towards the end of the talk is our inefficiency of doing the cloning procedure. Not just in our laboratory, but in all the laboratories right now. We still haven't come up to the efficiency that Mother Nature has. So we should be able to take our resources and focus on other conservation efforts. And, again, I agree on that, also.

We should focus on natural breeding. One of the things when I give talks to particular producers, and so forth or what we call farmers (Laughter) I mean, I grew up in the city, so they're farmers to me but they like to be called producers. (Laughter) Their biggest concern is that their livelihood is on natural breeding, and producing a better stock, and so forth like that.

And why would they want to use cloning technology? And, I can tell you right now: Cloning will never take the place of natural breeding. Because even though a producer has what he considers an elite cow, and he wants maybe five or six copies of this animal for his milking herd, he'd be foolish to consider having a whole herd of these animals. Because a disease can come through that this animal may be susceptible to, and it's going to wipe out their total herd.

Also, his I was going to say his farmer friend. But his friend down the road may be still doing natural breeding, and may produce a better cow two years down the line, and now *he* has the top elite cow in the country. And this guy is stuck with a herd of what, basically, would be considered genetic junk in his eyes. So it'll never take the place of natural breeding. And, of course, clones can't be reintroduced into the wild. That's a kind of people have been talking about.

But I think, eventually, it's not the clones that are going to be reintroduced, but, potentially, their progeny that can be introduced. Also, another concern is, little is known about the reproductive function of a lot of endangered species. So how can we, technically, clone these animals, if we don't even know about the basic reproduction? And, again, I agree on that, also.

So cloning is not going to save endangered species itself the technology and it will not bring back extinct species right now, as far as I'm aware of. It may, down the line but, again, this brings up a whole issue of ethical issues that we can discuss later.

How I'd like to see cloning technology be applied to endangered species is not to produce a group of identical individuals. Again, we don't mean, these animals are endangered. They're suffering from habitat loss. And, even in captivity, there's, potentially, a lot of inbreeding and so forth. So why clone them, in a sense, to make even more identical offsprings?

But how I see, or I'd like to see, cloning technology become useful is in salvaging lost genetics from populations. So, in other words, it may be possible particularly as zoos, and The Museum itself, start to bank tissue samples from species and I'm not talking about even endangered species right now I'm talking about just common species that are out there. As that species may become critically endangered, and so forth, genetics are potentially lost. We may now be able to go back to these frozen stocks, or frozen zoos, thaw out a cell line and salvage a genetic stock that would have been lost forever.

Also bring new genetics into a closed population. And this, particularly, becomes important either in fragmented populations in the wild or in captive populations in zoos. And the thing that I talk about is, if a zoo says or an organization needs to bring in new stock, it usually may require either if they don't have semen frozen back, or something like that or eggs or embryos they may sometimes have to bring in new individuals, either from other zoos or from the wild. It may now be possible to just actually bring in a tissue sample; make the animal in the laboratory; grow it up; and then, eventually, use that genetic into the stock.

This also brings up an issue of bringing in new genetics particularly with diseases. Some animals in the wild are infected with diseases tuberculosis, or even, now, what's going on in Europe, with the foot-and-mouth, like I discussed in our lunch. It maybe be possible to clean up cell lines in the laboratory. So, in other words, we can take tissue samples from an animal that would be infected with a disease and which would not normally be part of a breeding program, because it can pass the disease on. We can then treat that cell line in the laboratory.

And this is not only just for bacterial disease, but, eventually, for viral diseases. Clean up the cell line or clean up, technically, the animal and then we now have new genetic stock, that would be available to use for breeding programs. We can still keep the animal alive, in isolation or so forth. And, actually, this type of work is ongoing right now, and we're collaborating with a zoo. I don't really want to say which zoo right now, but that has animals that are infected with a viral disease. And we are attempting to clean up the cell line, and eventually apply that technology.

I think that cloning should be used, or can be used, as an assisted reproductive technique just as we used artificial insemination, embryo transfer, and embryo and semen cryopreservation. Eventually, I would like to see it used as an applied or an assisted reproductive technique particularly for species that don't breed well in captivity. Again, it's not going to replace natural breeding, but it would increase our understanding of the reproductive physiology of certain species. Particularly because, as we do the cloning technology, we find that there are some things that are not compatible.

And if we want to make this successful, we're going to have to do a lot more research on that particular given species. So we may, actually, be able to find out more information on the reproductive physiology. And then we might not have to use the cloning technology. Whatever new information we have, we may then be able to start looking at estrus synchronization, or even just going back

down to artificial insemination. And then to evaluate new applications of cloning. Like I mentioned before, using it for cleaning up diseases, and so forth.

Why don't we use cloning technology right now with endangered species? And, again, these are all the reasons why we or, these are all the steps that we need to do for cloning. And when you're talking about endangered species, there's a lot of these parts that are missing.

Sources of ovaries. Okay, we get them, like I said, from the slaughterhouse. The only way that we would be able to get them from a species in captivity, or a nondomestic animal, would either be from transvaginal oocyte recovery. That's been done before, particularly in the gaur. The Henry Doorly Zoo has been doing this for a while. We would be able to salvage them from animals that have been deceased. But the problem is that we need a lot of oocytes to do the cloning procedure right now, because it's not a very efficient procedure.

Again, oocyte maturation a lot of those techniques haven't been worked out for a lot of the species in captivity. And then, even, oocyte activation. I'm working now with domestic species, and we had to start from scratch. We had to start an oocyte-maturation system, we had to start an oocyte-activation system, and we needed a new embryo-culture system. I've been doing it for, now, over a year. We are finally, now, at the stage where we can produce embryos and transfer them. So, again, this is not a cure-all for endangered species right now.

What we did for the gaur project because we knew that we could not use gaur eggs, or transfer embryos into gaurs we used a technique what we call interspecies nuclear transfer. And this is not something that we developed in our laboratory it's been published before. There's been other attempts at it. Particularly Dominko, et al. Tanya's actually a friend of mine. She now does reside at Advanced Cell Technology. She used a bovine oocyte, and then fused cells from other species into the bovine oocyte, to see if she can get embryo development. And then she then transferred into the appropriate species, to try to get a pregnancy. So she looked at fusing mouse cells to bovine eggs pig, sheep and so forth.

Ken White, from the University of Utah, used common sheep eggs. And then used this wild-sheep species that is, I guess, endangered in, I believe, China. Again, he produced embryos and then tried to transfer them. So embryonic development was reported in both of these studies, but no fetal and initiation of pregnancy was at least claimed in the White, et al. Based on an ultrasound examination in which they saw fluid, but they could not recover any fetal tissue, and no live births were reported.

So our objective for this project was to evaluate if early and late fetal development could occur following interspecies nuclear transfer. And the overall cloning procedure was, we had a donor cell from the gaur. It came from a cell line that was frozen back at The Center for Reproduction of Endangered Species at The San Diego Zoological Society.

We used a recipient oocyte which was the bovine blastocyst and these were all in vitro matured. The recipient animal that we used was the cow the domestic cow, blastocyst. And the idea was to bypass the whole gaur system, in a sense never having to touch a living gaur to try to produce this offspring.

The nuclear transfers were the standard protocols that I mentioned. We cultured them in ACM, which is our embryo-culture media and we used irradiated mouse-feeders, just to help them along. Blastocyst development was evaluated on day seven and day eight, and then they were transferred at TransOva Genetics, which had a it's a large embryo-transfer company in the United States, in Iowa. That's where I did some research when I was a grad

student. A very, very small town I would not recommend going out there. But (laughter) all the embryos were transferred at their facility.

So some of the results. We produced 692 attempts at nuclear transfer, so we call these NT units. And this was done over a replicate of seven days. Out of those 692, only 235 cleaved that was about 34%. Only 12%, or 81 of them, became blastocysts, and these are some of the blastocysts right now.

We then transferred 44 embryos into 32 recipients, which means some embryos, two embryos were put into one cow. Only eight pregnancies were detected. Two were removed at about 46-54 days; four aborted early on in development; one late-stage abortion, about 200 days of gestation; and then one live birth was recorded at 293 days.

The fetal development. The reason why we sacrificed some of the fetuses was, once we started getting pregnancies, we wanted to make sure that we were able to see that they were normal development. And these are some of the fetuses. These are the chromosomes. We did karyotypes. We wanted to make sure that the nuclear material was gaur, and this shows that they are gaur. Gaur have 58 chromosomes; bovine have 60.

The cell lines were then we used nuclear markers. Again, this is bovine; and then this is the original gaur cell line; and these are some of the fetuses. And they, again, show that they were gaur nuclear material. So we actually did have clones of the original gaur. However, when we looked at the mitochondrial DNA, which is maternally derived, we actually found out that it was of bovine origin, not the gaur origin. So these are just some of the slides that show this is the bovine. It's digested. And you can see all the fetal tissue that we evaluated was bovine.

I'll skip this. This again, also, just shows-- The gaur was delivered at about 293 days, by caesarian. There were no abnormal pregnancies. We induced parturition. The animal was born in January of this year; died at about two days after birth. And an autopsy found out that he died from a bacterial infection from clostridium. We're still not sure where the source of clostridium came from. This is Noah, right after he was born they're cleaning him up.

These are some of the inefficiencies of cloning, as to why we're not really using this technology right now, and some of the problems that we're seeing even in domestic animals. Inconsistencies from the donor cell lines, all the way down to low embryo production. And low calf viability, low pregnancy rates.

We see a lot of abortions in cloning. Right now, about 50% of our pregnancies are aborted early on. We're evaluating research as to why this is occurring, and it seems to be a placental inefficiency. We sometimes see large calves. Again, we're not sure why, but we're evaluating that.

And because of these problems that we're seeing, new research is coming up. And what we're doing is, we're focusing on new ways of trying to improve oocyte maturation. And even by culturing follicles now, this would be particularly interesting when we're working with endangered species. We're improving the maturation of the oocytes, to try to see if that would improve our development rates and then, also, our pregnancy rates. We're also evaluating new activation systems and then, in culture conditions. And new embryo-transfer procedures. Particularly if we're going to use this in other species.

So, to conclude, we're able to prove that interspecies nuclear transfer can result in a live offspring, and that the mitochondria was capable even though it came from bovine oocytes was capable of directing development. Cloning is powerful technology, but it does have bugs, and further research is necessary to work out these bugs and before we can apply it to endangered species.

I just want to acknowledge ACT, TransOva Genetics, and the other labs that were associated with the project.

Thanks a lot.

(Applause)