

A Review of Cloning in the Horse

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More than 10 cloned foals were produced by two laboratories in 2006, and cloning is currently being offered commercially. Cloning is unlikely to result in individuals that excel in performance, but it can be used to produce breeding animals to help preserve valuable equine genetics. Author's address: Departments of Veterinary Physiology and Pharmacology and Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843; e-mail: khinrichs@cvm.tamu.edu. © 2006 AAEP.

1. Introduction

Equine cloning has been discussed in the popular press and in equine magazines since the birth of the first cloned equids (three mules and one horse) in 2003. In general, interest has been centered on whether or not the cloned offspring will be normal, how closely they will resemble the donor animal, and what cloning may be used for within the industry. Although equine cloning is still in its infancy, sufficient information is available from other species and from the few equine clones already produced to allow us to start answering these questions. This review will address the most common areas of concern to equine practitioners regarding equine-cloning procedures and outcome.

2. Technique

The principle of cloning is relatively simple. The chromosomes of a cell from the donor animal are transferred into the cytoplasm of an egg, and the egg is signaled to develop into an embryo. The cells from the donor animal are typically grown from a small (~5 mm³) sample of subcutaneous connective tissue. The tissue sample may be obtained through a small skin incision using sterile surgical technique. The tissue is placed in cell-culture medium,

cooled, and transported to the laboratory. At the laboratory, the tissue is placed into culture, and fibroblasts are grown from it onto the culture dish. The fibroblasts will proliferate until they cover the bottom of the plate (form a monolayer); at this time, they may be resuspended in medium and used to "seed" additional dishes. After a sufficient number of cells is obtained, the cells are typically frozen to be used at a later time.

Oocytes used for cloning may be harvested from the dominant pre-ovulatory follicles of live mares or they may be obtained by maturing immature oocytes in vitro. When mature (in metaphase II), the oocyte is enucleated by removing the metaphase plate and polar body using a small-diameter pipette through micromanipulation. The donor cell is then combined with the enucleated oocyte either by electrofusion or by directly injecting the cell into the cytoplasm of the oocyte. The recombined oocyte is activated to stimulate embryonic development; this is typically done by triggering calcium oscillations within the oocyte that mimic those that occur at fertilization.

After the recombined oocyte has been activated, it may be transferred surgically to the oviduct of a

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recipient mare, or it may be cultured in vitro to the blastocyst stage for transfer directly to the uterus of a recipient mare as for standard embryo transfer.

3. History of Equine Cloning

The first equid produced by cloning was born in 2003. This was a mule resulting from nuclear transfer of cells obtained from a fetus in 2003.¹ This mule (and two others born from the same cell line that year²) was produced using oocytes recovered ex vivo from mature follicles. After enucleation and recombination with donor cells, the oocytes were transferred surgically to the oviducts of recipient mares. The three mule foals resulted from transfer of >300 oocytes.

Also in 2003, a cloned horse foal was produced by the laboratory of Dr. Cesare Galli in Italy, and it resulted from nuclear transfer using a cell from an adult mare.³ This foal was produced using in vitro-matured oocytes and in vitro culture of the resulting embryo to the blastocyst stage, at which time it was transferred transcervically to the recipient mare. Seventeen blastocysts were transferred to produce the one foal. An interesting twist in this report was that the donor cell used to produce the foal came from the mare that ended up carrying the foal to term, which seems to contradict the hypothesis that pregnancies are more successful when the fetus is genetically different from the dam.

No cloned foals were reported in 2004. In 2005, two cloned foals were born in Dr. Galli's laboratory; one died within 48 h as a result of septicemia, and the other developed normally.⁴ Over 100 embryos were transferred to recipient mares to produce these foals. Also in 2005, our laboratory at Texas A&M University produced two cloned foals after transfer of 11 embryos.⁵ Both of the foals were healthy and are developing normally.

A large number of cloned foals were expected in 2006. Work in our laboratory at Texas A&M resulted in the birth of 9 live foals, of which 7 survived. A commercial cloning company, ViaGen, announced last fall that they had over 30 mares pregnant with cloned foals.⁶ Two viable foals have been announced by ViaGen at the time of writing (July 2006).

4. Health of Cloned Foals

Of the 16 live cloned horse foals known at the time of writing, we are aware of only 3 foals that have died post-partum [from septicemia (48 h), pneumonia (48 h), and complications associated with anesthesia for surgery to explore a possible bladder tear (4 days)]. This suggests that cloned foals may not suffer to the same degree from problems that are seen in other species. The three mule foals were also healthy. Since they were produced from a fetal cell line, which is more likely to produce viable offspring, their condition may not relate directly to what could be expected of an animal cloned from the cell of an adult horse.

In other species, notably sheep and cattle, cloned pregnancies tend to be lost throughout gestation,

and cloned offspring may suffer from fetal overgrowth and abnormalities of the heart, lung, and other organs.⁷ Abnormalities seen in cloned offspring are thought to be related to the failure of the DNA of the donor cell to be "reprogrammed" by the oocyte cytoplasm. This terminology refers to the process of changing the function of the donor cell DNA from that supporting a skin cell to that supporting the development of an embryo; the exact mechanism underlying this is currently unclear. In the donor fibroblast, only those genes needed for fibroblasts to function are being used. All other genes are turned off. Inhibition of gene function is obtained largely by modification (methylation or acetylation) of the DNA base molecules or of the histones (the proteins around which the DNA is wrapped). Because these modifications change the function of the gene without changing the gene itself, they are termed epigenetic modifications. The oocyte is somehow able to change the epigenetic markings of fibroblast cell DNA to those compatible with the growth of an embryo.

In theory, the more thorough the reprogramming at the time of nuclear transfer, the further in development the embryo will go. The high rate of early pregnancy loss reported in most of the studies on horse cloning indicates that reprogramming in the horse, as in other species, does not always occur effectively. However, because the majority of cloned foals born so far have been viable, it seems that the degree of reprogramming needed for an embryo to go to term is likely to produce a viable neonate. Information must be obtained on more cloned foals before we can definitively state the incidence of abnormalities in this population. The problems that we are aware of in cloned foals are the increased incidence of crooked front legs, which may be self-correcting or may require bandaging or splinting, and large umbilical remnants which may require surgical removal. Foals may initially be weak or show signs of maladjustment. Increased size of umbilical vessels is common in cloned calves, and the umbilical stump is typically removed prophylactically to prevent infection.⁷

A common question relating to the health of cloned foals is whether or not they will age prematurely. This thought stems from publicity around Dolly, the sheep that was the first clone of an adult mammal. Examination of Dolly's cells showed that the telomeres, the non-coding regions on the tips of the chromosomes that protect the chromosomes during replication, were shorter than normal for her age. Because the telomeres shorten with each cell division, they are longer in younger animals and shorter in older animals. The press wondered if Dolly was aging prematurely. In fact, Dolly died at 6 yr of age but not from "premature old age." Rather, she contracted a viral lung tumor, as did many others in her flock, while being housed indoors, and she had to be euthanized. The condition of short telomeres was later related to the source of

the cells used for cloning; mammary gland cells, like those used to produce Dolly, were found to result in calves with short telomeres, whereas fibroblasts resulted in calves with normal-length telomeres.⁸ The normality of telomere length in animals derived from fibroblasts has since been confirmed in several species.⁹⁻¹¹

5. How Closely Will the Clone Resemble the Donor?

A variety of factors will affect the degree of similarity between the cloned offspring and the original donor, but only two are actually related to the cloning procedure. Epigenetic changes compatible with a viable foal may still cause the gene function of a cloned foal to differ somewhat from that of the donor; therefore, the foal may potentially be shorter or taller, have more or less bone, etc. than did the donor animal. The second cloning-related potential cause of differences between the clone and the donor animal is related to mitochondria. The cytoplasm of the oocyte is filled with the mitochondria needed for oocyte maturation and embryo development. The embryo is formed from the oocyte, and thus, the mitochondria of the oocyte become the mitochondria of the offspring. The vast majority of mitochondrial function is governed by genes included in the nuclear DNA (~3000 genes), but in addition, they also have their own small DNA molecule coding for ~13 proteins and some functional nucleic acids. The mitochondrial DNA molecule in cloned foals will be derived from the host oocyte rather than from the donor fibroblast, or the foal may have a mixture of the two; therefore, the mitochondrial genotype of the cloned foal will differ from that of the donor. It is unknown whether or not this will produce any differences in phenotype.

If the cloned embryo was cultured *in vitro* before transfer to the recipient mare, *in vitro* culture itself has been shown to cause differences in neonatal size and other phenotype differences in other species. Little information is available in this area in the horse, because few foals have been produced from *in vitro*-cultured fertilized embryos.

Other potential causes of differences between the cloned foal and the donor would be seen in any transferred embryo; however, they will be more obvious in cloned foals because the expected phenotype is known. One of these causes is simply environment. Differences in uterine blood supply, placental sufficiency, problems occurring during birth, milk supply, dam and foal nutrition, handling and exercise regimens, vaccination and deworming programs, and training can all lead to differences in behavior or phenotype of the adult horse. In addition, individual differences arise during fetal growth; the most vivid of which are differences in white markings. As has been seen in cloned Holstein cattle and in the few identical twin foals produced by embryo splitting, there is a genetic "signal" for a white marking to occur on a given body part, but the migration of these white cells during fetal

growth is somewhat random. We have seen foals from a single genotype have one, two, and three white socks and have variations in face markings from a broad blaze to a star, stripe, and snip.

These variations in phenotype and in mitochondrial genotype will be useful in identifying individual cloned offspring that are produced from the same genetic donor. The possibility of phenotypic variation in cloned offspring as well as possible health problems associated with cloned neonates makes it unlikely that the cloned offspring will perform at the same level as the donor animal.

6. How Closely Will the Progeny of Cloned Animals Resemble Those of the Donor?

Herein lies the strength of cloning as a clinical procedure. Although no information is available in this area in the horse, in all other species studied, the progeny of cloned animals are normal. Even if the cloned animal itself has some epigenetic differences from the original donor, the epigenetic status of the DNA is reset when gametes are formed. Thus, the cloned animal's progeny should not differ from progeny of the original animal.

This is especially true in the case of stallions, because even the possibility of mitochondrial differences is eliminated. A cloned stallion will possess the mitochondrial genome from the oocyte used to produce it, and this genome will be present in mitochondria in its sperm. However, the sperm mitochondria are eliminated from the embryo after fertilization; the foal carries the mitochondria of the oocyte (the mitochondria of the dam). For this reason, the progeny of a cloned stallion should not differ from the progeny the original horse would have produced.

In the case of a cloned mare, the clone will produce oocytes carrying its mitochondrial genome, and therefore, its foals will also have this mitochondrial genome. In this way, they will differ from foals produced by the original mare. Again, it is unknown whether or not differences in mitochondrial genome will translate into differences in phenotype.

7. Is Cloning Clinically Feasible?

Although earlier work in cloning, as noted above, was inefficient (i.e., one foal from transfer of 100 embryos⁴), more recent work has shown a reasonable efficiency. For the foals our laboratory produced in 2006, we had ~5% blastocyst development from recombined oocytes, resulting in ~1-2 embryos ready for transfer per week. Transfer of 26 embryos resulted in the 9 live foals born this spring. The report of the commercial cloning company that >30 mares were pregnant with foals from six genotypes⁶ also suggests good efficiency, although the number of embryos transferred was not given. However, at the time of writing, only 2 live foals had been announced. Currently, companies have advertised horse cloning commercially at fees from

\$150,000 to \$370,000; these will almost surely decrease over time as efficiency increases.

8. How Will Cloning Affect the Industry?

Cloning is most accurately viewed as a method for genetic banking, similar to freezing semen or oocytes (although oocyte freezing is not yet effective) so that progeny may be obtained from a genetic line after the original horse is no longer fertile or is deceased. However, cloned horses are currently not eligible for registration with most breed registries in the United States. The Jockey Club only registers foals conceived by natural cover. The American Quarter Horse Association has specific wording in its rulebook that denies registration to any foal produced by a cloning procedure. Obviously, these rules are subject to change if the industry feels it is in their best interest. The European studbook, Zangersheide (a member of the World Breeding Federation for Sport Horses), will register cloned horses and has registered two of the cloned foals so far produced; this allows the offspring of these cloned animals to compete at official competitions in Europe.

Even in the United States, cloning is currently applicable to horses in which the value of the progeny does not depend on registration with a breed association. Thus, cloned animals may produce progeny that could compete in dressage, jumping, cross-country, polo, cutting, reining (i.e., in National Cutting Horse Association or National Reining Horse Association events), or other events.

The possibility exists with cloning for misuse and manipulation, and it is difficult to predict the range of these potential problems. The cloned animals themselves will be different from each other and from the original donor by their markings and their mitochondrial genotype (and, of course, from the donor by their age). It is our recommendation to microchip all cloned progeny for rapid identification. Cloning of a champion horse to produce a competitor to "ace the competition" is contrary to the guiding impetus of most breeders (that is to see what the next generation brings), but with huge purses to be had, some will surely consider this option. However, not only is cloning inefficient and costly, but it is also unlikely to produce a champion of the same quality as the original horse because of the various factors potentially affecting the performance of cloned foals. In addition, the cloned animal represents older genetics that are hopefully being improved on, and even at its best, it may not be competitive with the current foal crop. Most importantly, nearly all producers will understand that the success of any individual horse is because of both circumstances and talent. If the clone of a champion horse competes and does not do well, it will not only detract from the value of the clone as a breeding animal, but it will also detract from the value of the original animal. This may be the most potent fac-

tor that will limit the campaigning of cloned animals in performance events.

Can the progeny of cloned horses be differentiated from the progeny of the other horses? Progeny of cloned mares will be different from progeny of the original mare by their mitochondrial DNA. However, progeny of cloned stallions may not be different from progeny of the original stallion. Substitution of semen from a clone for semen from the original stallion would need to be monitored by evaluating the mitochondrial DNA from the semen sample. The small number of mitochondria in a spermatozoon presents some problems for efficient genotyping; this is an area that is currently under investigation at our genetics laboratory at Texas A&M University.

9. Summary

Equine cloning is possible today, and its value to the industry will be determined over the next few years. Cloning should be viewed as a method for producing a breeding animal rather than as a means to "duplicate" a performance horse. To the equine practitioner, cloning provides a procedure that may be offered to clients to preserve valuable genetics in the face of reproductive problems that in the past were insurmountable.

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