PERSPECTIVES: CLONING

Pigs Is Pigs

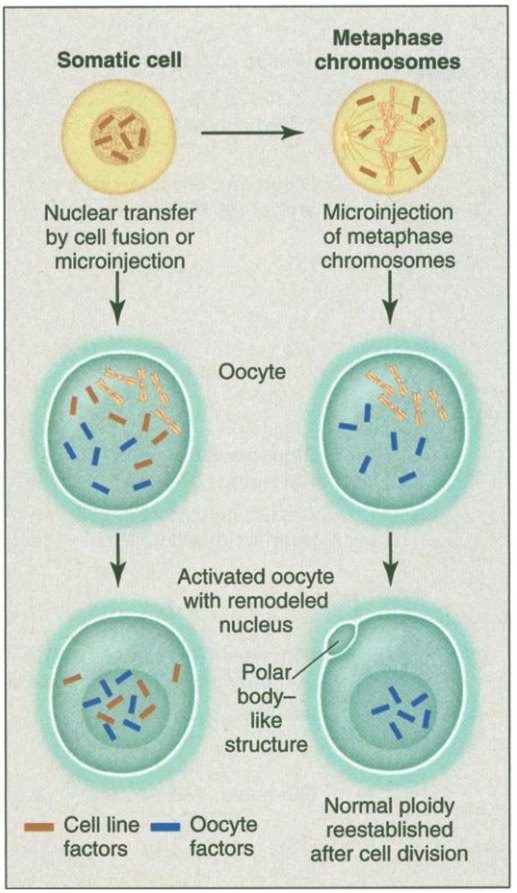
Randall S. Prather

Cloning mammals has become something of a cottage industry ever since Dolly first burst on the scene 3 years ago. With the same nuclear transfer technique that spawned Dolly—in which a cultured differentiated somatic cell is fused with a mature egg (oocyte) whose genetic material has been removed (enucleation)—births of live cloned offspring have been reported for sheep, cattle, and goats. However, the animal that is highest on everyone's list to clone but where cloning attempts have met with little success is the pig. Unlikely as it may seem, pigs are physiologically very close to humans and so there has been intense interest in using cloned pigs as organ donors for transplantation to humans (xenotransplantation). Finally, success has been achieved by Onishi *et al.* (*1*), who report the birth of a live cloned piglet called Xena, and Polejaeva *et al.*, who recently delivered five healthy cloned piglets (*2*).

There are two major problems facing pig cloners: activating development of the oocyte after nuclear transfer, and the need for at least four pig embryos in the uterus of a surrogate mother sow for embryonic development to proceed normally. In their approach, Onishi and colleagues (*1*) favored a technique termed piezo-actuated microinjection (in which a vibrating needle cleanly pierces the donor cell and oocyte plasma membrane), perfected by the Hawaiian team that reported the first cloned mice 2 years ago (*3*). The Onishi group directly injected porcine fetal fibroblast donor nuclei into enucleated oocytes. They used mature oocytes that were activated with one electrical impulse after nuclear transfer by microinjection. In a marked departure from the traditional nuclear transfer method, Polejaeva *et al.* (*2*) first fused porcine granulosa-derived donor cells with enucleated mature oocytes. After 18 hours, the donor nucleus was removed from the first oocyte and transferred to the cytoplasm of a fertilized egg. The investigators adopted this double nuclear transfer strategy because they surmised that in the original one-step method, the activation stimulus provided after nuclear transfer (designed to simulate the signal provided by the sperm at fertilization) was insufficient to support full-term development of the embryo.

Both groups used mature oocytes collected directly from female pigs rather than culturing immature oocytes in vitro. But here the similarities between the two studies end. A principal difference is the use of microinjection versus cell fusion for transfer of the donor nucleus to the enucleated oocyte. With microinjection, the plasma membrane and much of the cytoplasmic material of the donor cell is left behind, and only remnants of cytoplasm are injected with the donor nucleus into the enucleated oocyte. In contrast, with cell fusion, all of the components of the donor cell (both nuclear and cytoplasmic) become part of the oocyte.

A key feature of cloning by nuclear transfer is that the donor nucleus must be reprogrammed by oocyte-specific factors so that it can direct the development of the embryo. The components within a somatic cell that are responsible for directing its differentiation—for example, transcription factors, histones, and nuclear lamins—are associated with chromatin, and their composition and abundance changes with the differentiation status of the cell. If the entire donor cell is fused with an enucleated oocyte, then those donor cell–specific factors are also transferred into the cytoplasm of the recipient oocyte and can block the ability of oocyte-specific factors to reprogram the nucleus. If the nucleus alone is injected, then only those factors that are associated with chromatin will be transferred, raising the likelihood that oocyte-specific factors will be able to reprogram the donor nucleus. Another approach that neither group uses is to microinject donor cell metaphase chromosomes alone, rather than the entire donor into the recipient enucleated oocyte (see the figure). This strategy should prevent the transfer of most donor cell–specific factors, which is likely to result in a greater degree of nuclear remodeling and reprogramming.



The saga of eggs and bacon. When a donor somatic cell nucleus is transferred by cell fusion or microinjection to an enucleated oocyte, factors in the donor cell cytoplasm that are specific for that donor cell are also transferred to the oocyte. **(Left)** These factors along with oocyte-specific proteins become incorporated into the remodeled donor nucleus when it forms after oocyte electroactivation. If too many donor cell–specific factors are transferred to the oocyte, oocyte-specific factors become "diluted" making reprogramming of the donor nucleus less likely. **(Right)** If donor metaphase chromosomes instead of a complete donor nucleus were to be microinjected, the resulting remodeled nucleus in the activated oocyte would be assembled with only oocyte-specific factors, and reprogramming of the donor chromatin would become much more likely.

Now that a few cloned piglets have been born through nuclear transfer from cultured cells (*1, 2*), the trick will be to improve efficiency. Clearly, successful activation of the oocyte and retention of sufficient oocyte-specific factors to restructure the donor nucleus are essential. But there are other events that are necessary for successful nuclear reprogramming, for example, retaining the correct pattern of histone acetylation and DNA methylation during in vitro culture of the donor cell line. Interestingly, although many donor cell lines were tried, the Polejaeva and Onishi groups achieved cloning success with only one of their respective cell lines. One possible explanation for the cell lines that did not work is that culture-induced methylation changes in these donor cells caused

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aberrant gene expression when their nuclei were transferred to enucleated oocytes. Obata *et al.* (*4*) have shown that the pattern of DNA methylation is altered in donor cells cultured in vitro and that the original methylation pattern cannot be restored by transferring the donor nucleus to recipient oocyte cytoplasm. For successful reprogramming of the donor nucleus, the entire methylation pattern must be faithfully recapitulated from the beginning. Thus, for cells to be useful as donors for nuclear transfer, it is imperative that their genes retain their correct methylation pattern during manipulation in vitro.

Another important area that needs further research to improve cloning efficiency is the development of defined culture conditions—for example, culture medium lacking serum proteins, the quality of which varies between serum batches. Defined culture medium should allow successful maturation of pig oocytes in vitro as well as early development of embryos before their transfer to the uterus of pregnant sows. Although the Onishi and Polejaeva groups used mature pig oocytes that did not need to be cultured to become competent, the best way to obtain large numbers of fully mature oocytes (which will be required if cloning is to be scaled up) is to culture immature oocytes in vitro. Recently, such a highly efficient, well-defined culture system has been developed, which has resulted in the successful maturation and fertilization of immature pig oocytes and the birth of seven live piglets per litter (a large number for pigs) (*5*).

Currently, the most useful application of nuclear transfer technology is to produce transgenic domestic animals for research, because embryonic stem cells for these animals are not available. In terms of the food industry, one goal is to clone pigs that, for example, do not have the protein myostatin (a negative regulator of muscle growth) in order to produce animals with increased muscle mass. There will certainly be bureaucratic hurdles to jump through in order for transgenic pork to become a supermarket reality.

From the perspective of pig-to-human xenotransplantation, cloning pigs without the porcine cell surface antigen α-1,3-galactosyl transferase will provide a source of pig organs for transplantation that should not be rejected by the human immune system. One major problem with transplanting pig organs to humans is the danger of transferring pig endogenous retroviruses (PERVs) into human patients, which raises the possibility of another retrovirus pandemic if the viruses mutate to adapt to their human hosts. A paper accompanying the Polejaeva work describes the infection of a human cultured cell line with PERV from pig pancreatic islet cells (*6*). When the pig islets were grown in immunodeficient mice, they continued to produce PERV, which then infected several mouse tissues. The potential for PERV transmission needs to be fully addressed if therapeutic xenotransplantation—for example, the transplant of pig islets into immunosuppressed human diabetic patients—is ever to become an acceptable treatment. It is possible that certain breeds of pig carry PERVs but do not transmit them to human tissue, which would make such animals particularly valuable as organ donors.

Nuclear transfer will remain the method of choice for creating transgenic domestic animals until embryonic stem cell lines for them become available. To make nuclear transfer efficient, we need to learn much more about the molecular events that control cellular differentiation and how these events can be reversed to reprogram a somatic cell nucleus so that it can drive embryonic development.

References

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PERSPECTIVES: NEUROSCIENCE

Regional Differences in Cortical Organization

Michael S. Gazzaniga

Understanding how the microstructure of each region of the cerebral cortex relates to its particular function is still in its infancy. Why is it that some cortical areas of the human brain handle the processing of language and speech, whereas others carry out higher order perceptual processes such as face recognition? Are the signals carried by afferent neurons to these various cortical regions processed in different ways? Are the neurons in each cortical area organized differently?

To answer some of these questions, traditional anatomists have given us their cytoarchitectural maps depicting differences in the density and size of neuron clusters in various regions of the brain. And thanks to modern neuroscience we now have maps that profile neurotransmitter release within specific cortical regions when they are activated by incoming signals (*1*). But a map that depicts the cellular and structural differences between corresponding cortical regions in the right and left brain hemispheres—which look identical anatomically but carry out totally different functions—has not yet been made. Enter Galuske and colleagues on page 1946 of this issue to remedy this oversight (*2*).

Using a carbocyanine dye in postmortem human brain tissue, these investigators examined differences in the neuronal organization of Brodmann's area 22—involved in the processing of auditory signals associated with human speech—between the two hemispheres (*2*). During language processing, area 22 in the left hemisphere, which is crucial for word detection and generation, is preferentially activated; area 22 in the right hemisphere, which helps to discriminate between melody, pitch, and sound intensity, is activated to a much smaller degree. The authors find that area 22 in both the left and right hemisphere is sprinkled with clusters of neurons, all of the clusters having the same size. However, neuronal clusters in area 22 of the left brain are spaced about 20% further apart and are "cabled together" (*3*) with longer interconnecting axons than clusters in area 22 of the right brain. Consistent with these dramatic findings are the recent results of Hutsler and colleagues (*4*). They show that in area 22 and other language regions in the left hemisphere, pyramidal cells in the 90th to 100th percentile for size were larger than the biggest pyramidal cells in area 22 of the right hemisphere. Here, too, the authors argue that the biggest cells have the longest axons and that these axons are able to convey information between critical language zones spread out through the left hemisphere.

The great classical anatomist Brodmann argued for the existence of cortical specialization in 1909 when he wrote: "The specific histological differentiation

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