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Targeted Genetic Modification: Xenotransplantation and Beyond

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Introduction

The 10th anniversary of the report of the first animal cloned from an adult cell (Wilmut et al., 1997) is an excellent time to look back, and to look forward. The ability to clone domestic animals from differentiated cells not only provided new insight into the processes of development, but also significantly impacted the ability to make targeted genetic modifications to domestic livestock. The early work of nuclear transfer focused on using early embryos as a source of donor nuclei because it was thought that more differentiated nuclei either were more difficult or impossible to reprogram. While the strategy of using early embryonic cells could result in the production of cloned embryos and offspring; expansion of the genotype would require serial nuclear transfer to result in a significant number of clones. In addition the ability to genetically modify the donor nuclei was very limited. The developmental envelope was pushed by transferring nuclei from cells that were cultured from progressively more differentiated stages of embryos and fetuses. The first step was to take an early embryo and culture cells from that embryo and then use those cells for nuclear transfer (Sims et al., 1994). Next it was shown that fetal-derived cells could be cultured and subsequently used to clone individuals (Campbell et al., 1996). Once it was shown that donor cells could be cultured prior to nuclear transfer, it followed that the donor cells could be genetically modified prior to the nuclear transfer and that cloned transgenic animals could be produced (Schnieke et al., 1997). The advantage of using this system for making genetically modified animals is that the exact nature of the genetic modification could be determined in the cultured cells prior to creating the animal. This was very important for domestic animals since embryonic stem cells, which have been so useful for genetic modification in mice, have yet to be isolated from any domestic animal, and it was thus not possible to make targeted modifications. In addition, pre-selection of the donor cells would provide the investigator an opportunity to confirm that integration had occurred and that significant expression could be detected in the donor cells before the animal was made.

With the ability to genetically modify the donor cells prior to creating the animal in-hand, both the ideas of transgenic animals created from donor cells selected for high levels of expression of the gene of interest, as well as targeted modification (knock-ins and knock-outs) now entered the realm of imagination. Examples of transgenesis in pigs that used the strategy of selecting the cells prior to cloning the animal include addition of the enhanced green fluorescent protein, hFAT-1, endothelial cell nitric oxide synthase (NOS3), thymidine kinase, and cytosine deaminase (reviewed by (Prather, 2006).

Xenotransplantation

The importance of the ability to selectively modify a genomic region is illustrated by those that want to perform xenotransplantation. The organs of pigs are quite similar in size and physiology to humans (Turk et al., 2004) and thus are an excellent candidate for xenotransplantation (Ibrahim et al., 2006). Unfortunately, there is a specific Gal epitope (galactose alpha 1,3 galactose linkage) on the surface of most pig cells; including kidneys and hearts. The gene and

enzyme responsible for the Gal epitope is alpha 1,3 galactosyltransferase (GGTA1). Humans have preexisting antibodies for the Gal epitope; possibly from exposure to bacteria which have the same sugar linkage on their cell surface. Thus when pig cells or organs are transferred to humans or non-human primates, this combination of the Gal epitope and the preexisting antibodies results in hyperacute rejection (within minutes) of the pig cells. However, it should be noted that fewer than 5% of pig islet cells have this Gal epitope and thus are less likely to be rejected by hyperacute rejection and more susceptible to rejection mediated by T-cells and macrophages (Hering et al., 2006). Nevertheless, for organs like hearts and kidneys it was concluded that to get past the barrier of hyperacute rejection it would be necessary to get past this Gal epitope. Various strategies were envisioned and acted upon. These included inhibiting the compliment system, masking the Gal epitope with other carbohydrates, and knocking-out GGTA1 (Ibrahim et al., 2006). It was hoped that by getting past the hyperacute rejection that the organ or cells would not be rejected until the host developed an acquired immunity. It was also hoped that this acquired immunity would be delayed and that during this delay tolerance to the graft might be induced.

The first attempt at making a domestic animal with GGTA1 knocked-out was in sheep, but it was only partially successful as all the knock-out animals died (Denning et al., 2001). Then in 2002 it was reported that pigs with one allele rendered non-functional could be made (Lai et al., 2002) and that with subsequent modification to the other allele (Kolber-Simonds et al., 2004) that organs from these pigs could survive for an extended period without signs of hyperacute rejection (Tseng et al., 2005; Yamada et al., 2005). With this barrier of hyperacute rejection now surmounted, other genetic modifications that will be necessary for overcoming acquired immunity could be investigated. We, in collaboration with scientists at Immerge Biotherapeutics have taken pigs of the Imutran line with a human decay accelerating factor addition (hDAF) addition and knocked out GGTA1 and are now distributing this model from the National Swine Resource and Research Center (http://www.nsrrc.missouri.edu). It is likely that other modifications will need to be made prior to the successful transfer of pig organs to humans. The important point is that now we have the technology to add or remove genes with precision, and it is thought that many of the obstacles to xenotransplantation can be overcome with additional genetic modifications (Ibrahim et al., 2006).

Other Biomedical Knockouts

While previous techniques, like pronuclear injection, permitted random insertions of genes, the concept of creating a genetic modification in a more precise fashion prior to creating the animal was not possible. Such ability now presents many other possibilities, as we still do not have good models for many human diseases. One such example is cystic fibrosis. The gene responsible for cystic fibrosis is the cystic fibrosis transmembrane conductance regulator (CFTR). Unfortunately, mutation of this gene in mice fails to reproduce the clinical disease that plagues humans with cystic fibrosis. Lung disease is the current cause of most of the morbidity and 95% of the mortality. Exocrine pancreatic failure is also an important clinical problem for patients. In contrast, mice develop neither lung nor pancreatic disease and instead die of intestinal disease. As with the cardiovascular system, pigs share important anatomical and physiological features that are more similar to humans, than mice are to humans. In collaboration with Dr. Michael Welsh at the University of Iowa we are currently working toward the goal of making pigs with both CFTR alleles disrupted via the most common ΔF508 mutation seen in humans as well as a complete knockout of the gene.

Other modifications that might be useful to pursue include knocking-out NOS3. In collaboration with Dr. M. Harold Laughlin at the University of Missouri we've already made NOS3 overexpresser pigs (Hao et al., 2006), and are working toward a knock-out model. We expect both of these modifications (the knock-out and insertion) to be useful for a number of

applications, the foremost of which is to study mechanisms in the pathogenesis and treatment of cardiovascular disease. In collaboration with Drs. Monique and Christian Lorson at the University of Missouri, another gene is being targeted to create a model of 5q-linked spinal muscle atrophy, a pediatric neurodegenerative disease. Currently there are no large animal models of this disease. In addition, there are a number of other possibilities for knocking-out genes in pigs that may prove to be useful for biomedical science as well as for agriculture.

Conclusions

While much of the focus of Dolly has been on the ability to clone from an adult cell and a new understanding of the plasticity of the program of the genome, there were other important spin-offs of the technology that led to Dolly. Knocking-in or knocking-out a gene would not have been possible without the early studies that showed that cultured cells could be used as donors to clone an animal. While there are still limitations to the process of targeted genetic modification; the foremost of which is the lack of a suitable stem cell, such as an embryonic stem cell in mice, upon which to make the genetic modifications. One of the most common cell types currently used are fetal-derived fibroblast cells. Unfortunately these cells undergo senesce after about a month in culture. Thus any genetic modification needs to be made immediately after isolation such that selection, expansion and cryopreservation can be accomplished within a month. Thus a next area of advancement in improving the efficiencies of creating targeted modifications in cells that can subsequently be cloned will be the characterization of domestic animal stem cells. These stem cells need to be plastic in that they are clonable at a high efficiency, and they will need to proliferate rapidly and grow for an extended period.

Much has been accomplished since the birth of Dolly and her kin. Other species have been cloned, and many genetic modifications have been made that have resulted in significant advances of our understanding of biology in general, and specifically as outlined above to xenotransplantation. Advancements in the cloning, targeted genetic modification, and cell culture procedures will result in significant impacts in all areas of biology.

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