

Use of Cloning and Transgenesis in Pigs

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Introduction

A brochure from the breeding company contains a countless number of animals from which to select breeding stock. A caption below one of the 'genetic prototypes' reads "just feed Tender-Gro® 30 days prior to slaughter and we 'guarantee' that animals fed this product will produce the most tender meat possible for your local packer, money back guarantee." Another caption reads, "feed Gro-Fast® to those animals (males or females) destined for meat production and we guarantee faster weight gains and maximal returns when animals are sold on a grade and yield program." Yet, another reads, this animal has been the top carcass animal "on the rail" for the last three years. Although the aforementioned seem somewhat futuristic in principle, or perhaps impossible in the case of the latter, recent developments in biotechnology and genetic engineering make all scenarios possible in the near term.

The exact mechanisms controlling calpain activity in postmortem muscle are far from being "well-established," yet many would argue that control of these proteases alone are key to making meat more tender in the future for consumers (Goll *et al.*, 1998). Many have shown that during postmortem ageing, calpains attack and degrade proteins that are important for maintaining the organization and structure of muscle proteins (Koochmaraie *et al.*, 2002). Once disrupted, muscle (meat) becomes more tender because less force is required during the mastication process. What would it be worth to the meat industry for such a crucial protein to be present, and active, in higher than normal concentrations in the muscle of cattle at the time of slaugh-

ter? Is it even possible to deliver such proteases to the muscle? If so, the goal of providing consumers with consistently palatable meat is within grasp.

Regulation of growth, especially lean growth, is a complex mechanism that is likely controlled by a myriad of physiological parameters. One such physiological parameter that augments whole body growth is circulating levels of growth hormone (GH). When exogenous growth hormone is administered to lactating cows, milk production is greatly enhanced. Of course, this whole process has been exploited by agriculture and has been successfully commercialized for improving dairy herd production (Bauman *et al.*, 1999). In other species, for example in pigs, the response is somewhat different. In particular, daily administration of growth hormone not only improves growth rate but also acts as a "repartitioning agent" whereby nutrients are directed away from adipose tissue deposition and toward lean body mass growth (Thiel *et al.*, 1993). As a result, altering circulating growth hormone levels is a very attractive means of improving growth performance and productivity as well as lean composition. Unfortunately, the requirement for daily administration of GH is not, however, feasible nor is it practical to many in the meat animal industries. Furthermore, there is substantial public resistance to using "injected hormones" as a means for improving animal productivity. Even though animal scientists must remain cognizant of public concerns, it is the obligation of those charged with improving the efficiency of growing animals to remain vigilant and receptive to opportunities that may augment growth in a "consumer friendly manner." This is clearly the benefit of using transgenesis and cloning. One such strategy currently being investigated rigorously to circumvent repetitive and constant delivery of growth hormone is to target "upstream" regulators of growth hormone secretion somatotrophs from the anterior pituitary gland. In particular, scientists at Baylor have successfully expressed enough growth hormone releasing hormone (GHRH) in pig skeletal muscle to increase circulating insulin-like growth factor I concentrations, which are "down-stream" of circulating growth hormone and elucidate peripheral tissue responses in the body (Draghia-Akli *et al.*, 2002). Furthermore, these pigs grew at a faster rate than controls. Although this study was conducted using electroporation of DNA in skeletal muscle, these data show that the strategy of targeting growth hormone releasing hormone as a means to improve growth performance via the GH axis is possible and suggest that

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this gene may be easily exploited using transgenic animal technologies.

Transgenic Animal Production

A cursory perusal of the biomedical literature will quickly reveal that transgenic animals, most notably transgenic mice, have contributed greatly to our understanding of how cells and organisms function. Both simple gene addition as well as gene removal has facilitated this addition to our knowledge base. The addition of genes permits questions to be answered about both gain of function and more recently reduction of function by knockdown experiments that use RNAi strategies (Tabara *et al.*, 1998). One of the most important additions to the biologist's arsenal has been the ability to knockout a gene (Smithies *et al.*, 1985). In one embodiment, by using this strategy a stop codon is inserted into the coding region of a gene. When the ribosome translates the resulting mRNA, the polypeptide is terminated and a shortened version of the protein is produced. If, for example, the production of the protein is terminated prior to the catalytic region of the mature polypeptide, then the function of that enzyme is knocked out. Alternatively, the gene can be altered to produce a modified protein, thus modifying function.

The actual knockout of a gene requires a technique entitled homologous recombination, and in mice, generally embryonic stem cells. A large number of embryonic stem (ES) cells can be used with either a conventional knockout strategy (both positive and negative selection) or by using a gene trap strategy. The specific recombination events are relatively rare (1 in every 1,000,000). The inefficiency of these techniques is not a problem because a large number of ES cells can be gathered to begin the project, and the ES cells can be maintained for a long period of time *in vitro* without undergoing differentiation or senescence. These two properties permit selection procedures that result in the survival of only those cells that have undergone the site-specific homologous recombination. These surviving cells can then be tested, and if appropriately modified, injected into the cavity of a mouse blastocyst. Inside the blastocyst, they form a chimera with the host inner cell mass cells of the blastocyst and result in a chimeric offspring. If some of these ES cells contribute to cells that form sperm or eggs, the genetic modification introduced by homologous recombination can be passed on to offspring, establishing that genetic modification in the mouse. To date, the establishment of functional embryonic stem cells that can form chimeras and contribute to the germ line has only been shown in the mouse despite numerous attempts in other species (swine, ovine, bovine) (Piedrahita, 2000, Wheeler, 2001).

Prior to December 2001, there were only a few methods described to make swine transgenic (Prather *et al.*, 2003). These included injection of DNA directly into the pronucleus of a 1-cell stage embryo (Hammer *et al.*, 1985), and sperm-mediated transfection via fertilization (Gandolfi *et al.*, 1989, Sperandio *et al.*, 1996). In December 2001, two addi-

tional methods were described, oocyte transduction (Cabot *et al.*, 2001) and transduction of fetal-derived cells followed by cloning via nuclear transfer (Park *et al.*, 2001). The major limitation of all these approaches for pigs is the lack of control over how many copies of the gene integrate into the genome, as well as where those copies enter the genome. Thus investigators were limited to the addition of genes, and it was not possible to remove gene function.

Simple gene addition has been very useful in swine for both production agriculture and medical research. This topic was reviewed in 2000 at this meeting (Wells, 2000). Data was presented that show that pigs that incorporated a variety of transgenes (IGF-I, growth hormone) had in some cases increased growth rates and increases in lean muscle mass. In addition to altering meat quality and efficiency of production, the addition of genes has been very useful for things like the study of eye diseases. One group at North Carolina State University has created swine with mutated forms of rhodopsin (Blackmon *et al.*, 2000; Petters *et al.*, 1997). These animals manifest disease similar to human retinitis pigmentosa. Testing treatments in pigs has saved human patients from potentially harmful clinical trials. Also the possibility of xenotransplantation of swine organs into humans has been pursued by a variety of investigators who have added genes to modify complement hMCP (Diamond *et al.*, 2001), hDAF (Cozzi *et al.*, 1997), H2-DAF/beta actin-CD59 (Byrne *et al.*, 1997, Levy *et al.*, 2000), and carbohydrates by competitive inhibition (Costa *et al.*, 1999) and blocking of Gal epitopes (Miyagawa *et al.*, 2001).

Thus while the addition of genes has proved very useful, the technique has limitations. In some cases removal of a gene is necessary. For example, if one wanted to determine if myostatin knockout in swine would result in an increase in lean muscle mass it couldn't be done by the random addition of a gene. A technique to modify the coding sequence of this gene such that a functional protein is not produced is necessary. In the example of xenotransplantation, gene addition has resulted in prolonging the life of pig organ in nonhuman primates, but removal of a specific molecule on the cell surface is still required. In order to remove gene function in pigs, since there are no ES cells as in mice, it is necessary to perform homologous recombination on the donor cells and then use those donor cells for nuclear transfer and cloning to create the animal (see below).

Knockout Swine

In January 2002, we published a technique that resulted in the removal of gene function (Lai *et al.*, 2002). This technique used a gene trap strategy on fetal-derived fibroblasts followed by cloning via nuclear transfer. The gene whose function was removed was alpha (1, 3) galactosyltransferase (GATT1). The galactose 1, 3 galactose sugar linkage produced by this enzyme is thought to be responsible for hyperacute rejection when pig organs are transferred into primates (Auchincloss and Sachs, 1998, Cooper *et al.*, 2002).

The only way to completely remove the function of this gene is to disrupt the coding region such that a functional enzyme cannot be produced. Two technologies came together to enable the production of these knockout pigs: 1) a quick method of making the genetic modification, and 2) the ability to clone those genetically modified cells by transfer of the nuclei to enucleated oocytes. The gene trap strategy followed by quick selection was necessary because a stable cell line such as an ES cell line in pigs is not available, and the fetal derived fibroblast cells senesce after about 30 population doublings. Thus isolation, homologous recombination (gene trap), selection, and expansion of those survivors had to occur rather quickly. If not performed quickly, the cells would senesce prior to use in the nuclear transfer procedures (Lai *et al.*, 2002). The second technology that came of age is the same technology that produced Dolly the cloned sheep (Wilmut *et al.*, 1997): cloning by nuclear transfer.

More recently, a second round of selection was performed on cells that had one copy of the GATT1 gene already removed (Phelps *et al.*, 2003). They discovered a single random point mutation that occurred in the reading frame of the other copy of the gene. These cells were expanded and used for nuclear transfer. Their domestic pigs now have both copies of the gene rendered non-functional. Similarly, we used our first GATT1 knockout gilt (NIH miniature pig) to derive fetal fibroblasts after nuclear transfer. We then added antibodies that recognize the galactose 1,3 galactose sugar linkage and compliment. Thirty-two clones were identified (~10⁻⁴) and one of these clones, after nuclear transfer and embryo transfer, resulted in a normal offspring (named Goldie) that did not have a functional copy of the GATT1. Neither human serum, baboon serum, nor IB4 lectin binds to the cells isolated from Goldie (Lai *et al.*, in preparation). Thus she is an excellent candidate for the production of organs that might be transferred into humans.

Swine as Models for Basic Research, Medicine and Agriculture

Genetically modified swine will have uses in both basic research as well as in production agriculture. In many cases the genetically modified mouse is not suitable for the studies at hand. In discussions with researchers who work on mice with specific genetic modifications, the issue of size repeatedly arises. Mice, in many cases, are simply too small to take measurements (e.g. coronary artery blood flow for cardiovascular studies) or to practice treatments (bone splinting for osteogenesis imperfecta). Children born with osteogenesis imperfecta have weak bones and the treatment of choice is splinting to repair the broken bones. The mouse model exhibits the correct phenotype, but is simply too small to practice the splinting technique (Forlino and Marini, 2000). Similarly, a mutation in Fibrillin 1 results in humans that are subject to aneurysms and has resulted in the deaths of athletes (Kielty *et al.*, 2002). Again, the knockout mice exhibit the phenotype, but are too small to test

treatment strategies. Finally, retina transplants have been conducted in rats (Klassen *et al.*, 2001), but even rat eyes are much smaller than human eyes and present challenges for developing treatment strategies.

In other instances, mice do not exhibit the expected phenotype. In the case of cystic fibrosis the CFTR is mutated and results in the lack of chloride ion movement across the membrane. This gene has been mutated in mice, but there is no airway disease phenotype (Grubb and Gabriel, 1997), i.e. mice have a compensatory mechanism. Thus, even though the mouse is too small to test many of the mechanical treatments that are used for humans that have cystic fibrosis, it also has no symptoms of the disease. Thus a knockout of CFTR in another species such as the pig is warranted.

Large Offspring Syndrome

A discussion of animals derived by nuclear transfer requires a few words about abnormal phenotypes in offspring derived by this technology. Generally, these aberrant phenotypes are referred to as Large Offspring Syndrome (LOS). LOS was first described in cattle that were derived from *in vitro* oocyte maturation, *in vitro* fertilization and culture prior to embryo transfer. The most prevalent phenotype is that of a skewed distribution of birth weights, with some of the offspring over twice the normal size (Walker *et al.*, 1996, Wilson *et al.*, 1995). The aberrant phenotypes are species specific: cattle show large birth weights and/or contracted tendons; mice show large placenta and/or obesity in old age; pigs show contracted tendons and/or respiratory problems. Fortunately, these phenotypes are not transmitted to the next generation (Tamashiro *et al.*, 2002, Conway, 1996, Carter *et al.*, 2002), as they appear to be a result of aberrant DNA methylation in the donor cell line or during early embryogenesis, and the DNA methylation pattern is erased and reestablished during gametogenesis (Humpherys *et al.*, 2001, Rideout *et al.*, 2001). Thus LOS is a management concern only in the first generation, as the aberrant phenotypes are apparently not passed on to the offspring.

Conclusion

While we now have the technology in-hand to add genes as well as remove genes, the procedures are not efficient. Technology that may be used in the near future to create pigs with specific genetic modifications is that of manipulation of the male germ cell prior to introduction into an animal that has had its germ cells depleted (Brinster, 2002). It may be possible to perform homologous recombination on the germ cells prior to formation of the sperm. Transplantation of these cells into a host may permit the production of genetically modified sperm cells. Then a male carrying these cells could be used to breed a large number of females and the resulting offspring would carry the genetic modification.

As previously stated in the introduction, the potential application of genetic modification to meat science is enor-

mous. The ability to make livestock grow faster and produce more meat that is more palatable is exciting and could revolutionize the animal industry. However, one of the greatest limitations to the development and propagation of transgenics, other than the low percentage of viable offspring, is the limited availability of known genes that are economically important. Moreover, tissue-specific promoters, or those DNA elements responsible for controlling expression of genes, are rather scarce and need further development. Currently, genes like the aforementioned and myostatin, which is responsible for the double muscled syndrome in cattle (McPherron and Lee, 1997), are the only genes that have been studied sufficiently to merit such an aggressive means of exploitation in the area of meat production. Of equal importance, however, is the fact that many of these "candidate" genes need to be expressed in a time and tissue-dependent manner. For example, myostatin is a negative regulator of muscle development (Lee and McPherron, 2001). In a mutated form, this gene product is incapable of controlling muscle development properly and thus yields a double muscled phenotype in cattle. Because the myostatin may modulate other physiological phenomena in other tissues, transgene expression needs to be directed or restricted to developing skeletal muscle. Furthermore, development of muscle fibers occurs over fairly narrow window of prenatal development. Therefore, the utility of using myostatin or a mutated form of this gene in transgenics would be greatly enhanced if transgenes were controllable. At present, there are a limited number of promoters available for expressing transgenes in a tissue-specific manner. The most often used promoter is the muscle creatine kinase (MCK) promoter (Jaynes *et al.*, 1988). Because MCK is expressed solely in muscle cells, this promoter is ideal for restricting transgene expression to muscle cells. This promoter has been used successfully to develop a number of transgenic mouse lines.

Controllable promoters, on the other hand, or those promoters that respond positively or negatively to various compounds, either fed or injected, have had limited success outside the cell culture environment. However, continued development of such "reagents" must occur if maximal benefits are going to be realized by transgenic approaches.

As a discipline, it is imperative that we continue to research and study, in detail, those biochemical and molecular processes that drive meat production. As a result of these efforts, we will continue to discover genes that may some day be used to generate transgenic animals for commercial meat production. Since the technology is now available to make most any genetic modification we are now limited only by our imagination and baseline data needed to justify the efforts.

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