

# Production of transgenic livestock: Promise fulfilled<sup>1</sup>

M. B. Wheeler<sup>2</sup>

Department of Animal Sciences, University of Illinois, Urbana 61801

**ABSTRACT:** The introduction of specific genes into the genome of farm animals and its stable incorporation into the germ line has been a major technological advance in agriculture. Transgenic technology provides a method to rapidly introduce “new” genes into cattle, swine, sheep, and goats without crossbreeding. It is a more extreme methodology, but in essence, not really different from crossbreeding or genetic selection in its result. Methods to produce transgenic animals have been available for more than 20 yr, yet recently lines of transgenic livestock have been developed that have the potential to improve animal agriculture and benefit producers and/or consumers. There are a number of methods that can be used to produce transgenic animals. However, the primary method to date has been the microinjection of genes into the pronuclei of zygotes. This method is one of an array of rapidly developing transgenic methodologies. Another method that has enjoyed recent success is that of nuclear transfer or “cloning.” The use of this technique to produce transgenic

livestock will profoundly affect the use of transgenic technology in livestock production. Cell-based, nuclear transfer or cloning strategies have several distinct advantages for use in the production of transgenic livestock that cannot be attained using pronuclear injection of DNA. Practical applications of transgenesis in livestock production include enhanced prolificacy and reproductive performance, increased feed utilization and growth rate, improved carcass composition, improved milk production and/or composition, and increased disease resistance. One practical application of transgenics in swine production is to improve milk production and/or composition. To address the problem of low milk production, transgenic swine over-expressing the milk protein bovine  $\alpha$ -lactalbumin were developed and characterized. The outcomes assessed were milk composition, milk yield, and piglet growth. Our results indicate that transgenic overexpression of milk proteins may provide a means to improve swine lactation performance.

Key Words:  $\alpha$ -Lactalbumin, Mammary Glands, Milk, Transgenic Animals

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## Introduction

The ability to insert genes into livestock embryos, the incorporation of those genes and their stable transmission into the genome of the resultant offspring will enable major genetic advances to be realized in animal agriculture. Production of transgenic livestock provides a method to rapidly introduce “new” genes into cattle,

swine, sheep, and goats without crossbreeding (Pursel and Rexroad, 1993). It is a more abrupt methodology, but in practicality, not really different from crossbreeding or genetic selection in its result. There are two basic strategies used when producing transgenic animals. These are the so-called “gain of function” or “loss of function” transgenics. The basic idea behind the gain of function paradigm is that by the addition of a cloned fragment of DNA to an animal’s genome, one can accomplish several objectives. One objective is to obtain new expression of a gene product that did not previously exist in that cell or tissue type. This is the type of strategy that was used for the animals discussed in this paper.

Some of the other methods that have been used to produce transgenic animals include: 1) DNA transfer by retroviruses; 2) microinjection of genes into pronuclei of fertilized ova; 3) injection of embryonic stem (ES) cells and/or embryonic germ (EG) cells, previously exposed to foreign DNA, into the cavity of blastocysts; 4) sperm-mediated exogenous DNA transfer during in vitro fertilization; 5) liposome-mediated DNA transfer into cells

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<sup>2</sup>Correspondence: 366 Animal Sciences Laboratory, 1207 West Gregory Drive (217) 333-2239; fax: (217) 333-8286; e-mail: mbwheelee@uiuc.edu

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and embryos; 6) electroporation of DNA into sperm, ova or embryos; 7) biolistics; and 8) nuclear transfer with somatic or embryonic cells (see review by Wheeler and Walters, 2001).

### Historical Overview of Transgenic Technology

The foundation for the production of transgenic animals was provided by the pioneering experiments of Brackett et al. (1971) using sperm-mediated gene transfer in rabbits. Although gene transfer was accomplished in that study, germ-line transmission of the transgene was not successfully reported until 1976 using retroviral gene transfer vectors in mice (Jaenisch, 1976). In 1980, Gordon et al. showed that pronuclear microinjection of DNA into one-cell mouse zygotes was a relatively efficient method to accomplish germ line gene transfer. Microinjection of cloned DNA into the pronucleus of a fertilized ovum has been and continues to be the most widely used and most successful method for producing transgenic mice and livestock (Hammer et al., 1985; Wall, 2002).

There have been a number of methods reported in the past 15 yr, which have successfully produced germ line transgenic animals from various species (see review by Wheeler and Walters, 2001). Since the famous cloned sheep "Dolly" was born (Wilmut et al., 1997), nuclear transfer technology has become another methodology available for the production of transgenic animals. These new methods for the production of genetically identical individuals from embryonic (Campbell et al., 1996; Wilmut et al., 1997) and somatic (Wilmut et al., 1997; Polejaeva et al., 2000) cells, via nuclear transfer, should allow the rapid development of genetically identical animals with a targeted gene insertion. These developments will enhance our ability to produce transgenic animals with genes inserted into specific sites in the genome. This method will most likely become the most useful method for future production of transgenic livestock. These nuclear transfer strategies have several distinct advantages for use in the production of transgenic livestock that cannot be attained using pronuclear injection of DNA.

### Applications for Transgenics in Livestock Production

Practical applications of transgenics in livestock production include improved milk production and composition, increased growth rate, improved feed utilization, improved carcass composition, increased disease resistance, enhanced reproductive performance, increased prolificacy, and altered cell and tissue characteristics for biomedical research (Wheeler and Choi, 1997) and manufacturing. The production of transgenic swine with growth hormone serves as an example of the value of this technology (Brem et al., 1985, Hammer et al., 1985).

Transgenic alteration of milk composition has the potential to enhance the production of certain proteins and/or growth factors that are deficient in milk (Bremel et al., 1989). The improvement of the nutrient or therapeutic value of milk may have a profound impact on survival and growth of newborns in both humans and animals. The enhancement of the nutrient or therapeutic value of milk may have profound impact on survival and growth of newborn pigs.

In many production species such as cattle, sheep, and goats, the nutrients available to the young may not be limiting. However, milk production in sows limits piglet growth and therefore pig production (Hartmann et al., 1984). In swine, 44% of the growth rate of the developing piglets can be attributed to yield and composition of the sow milk (Lewis et al., 1978). Methods that increase the growth of piglets during suckling result in an increase in weaning weights, a decrease in the number of days required to reach market weight, and thus a decrease in the amount of feed needed for the animals to reach market weight.

The high percentage in growth rate attributed to milk indicates the potential usefulness of this technology to the developing piglet. An approach to increase milk production in pigs may be accomplished by alteration of milk components such as lactose, a major osmole of milk in mammary gland cells. The overexpression of lactose in the milk of pigs will increase the carbohydrate intake by the developing young, resulting in improvement of piglet growth.

The overall result of the transgenic modification of milk will be the creation of more uses of milk and milk products in both agriculture and medicine. Increasing the concentrations of existing proteins or producing entirely new proteins in milk is truly a "value-added" opportunity for animal agriculture.

### Transgenic Animals with Specific Gene Expression in the Mammary Gland

Numerous laboratories have studied the expression of transgenes specifically in mammary tissue (Simons et al., 1987; Vilotte et al., 1989; Clark et al., 1989; Bleck and Bremel, 1994; Bleck et al., 1995, 1996; Bleck et al., 1998). The 5' flanking regions of many milk protein genes, which have a regulatory function, have been used to drive expression of foreign proteins in mammary epithelial cells of transgenic animals (Simons et al., 1987; Vilotte et al., 1989). Regulatory regions of milk proteins have been linked to genes that have been expressed as transgenes in a variety of animals (pigs, sheep, and goats; Clark et al., 1989; Ebert et al., 1991; Wall et al., 1991). Levels and patterns of expression have been very similar to those observed in numerous transgenic mouse experiments. These regulatory regions have shown little or no species specificity and have even been regulated properly in species that do not express those proteins (Wall et al., 1991; Simons et al., 1987).

Something as complex as milk production is controlled in part by counterbalancing factors (homeostatic control), where altering expression of one gene may be counterbalanced by endogenous expression of another. This means that identifying single-gene transgenic approaches to enhancing production characteristics is a difficult challenge. Understanding the biological control of a production characteristic before making the tremendous investment in developing transgenic animals is of great importance.

Previous work has suggested that the volume of milk produced is directly dependent upon the amount of lactose synthesized. Lactose is synthesized in the Golgi apparatus of mammary secretory cells by the lactose synthase complex. This complex is composed of the mammary-specific protein  $\alpha$ -lactalbumin and the enzyme  $\beta$ 1,4 galactosyltransferase. The reaction catalyzed by lactose synthase is  $\text{UDP-Galactose} + \text{Glucose} \rightarrow \text{Galactose-Glucose (Lactose)} + \text{UDP}$ .

Lactose is formed inside the secretory vesicles of the mammary Golgi (Brew and Grobler, 1992). These vesicles are budded off from the Golgi complex, transported to the apical membrane of the epithelial cell, and secreted into the lumen. Because lactose cannot diffuse out of the vesicles, it acts to draw water by osmosis into the vesicle. Since lactose synthase is necessary for the production of lactose and the movement of water into the mammary secretory vesicles and then into the lumen of the gland, it is critical in the control of milk secretion (Hayssen and Blackburn, 1985). There is evidence that suggests that milk volume is directly related to the expression of the  $\alpha$ -lactalbumin gene.  $\alpha$ -Lactalbumin is a normal constituent of milk, and its expression correlates with the induction of copious milk secretion at the onset of lactation (Goodman and Schanbacher, 1991).

Of all the bovine milk protein genes, the expression of bovine  $\alpha$ -lactalbumin is the most lactation-specific and strictly controlled (Goodman and Schanbacher, 1991). The unique expression pattern of the bovine  $\alpha$ -lactalbumin gene makes its promoter and regulatory elements an attractive choice for a mammary expression system in transgenic animals.

### Improvement of the Lactation Performance in Sows

Large increases in average milk production of dairy cattle have been realized over the past several decades because of intense selection for milk yield, which is a trait that is easy to objectively measure. Similarly, milk production by sows has increased over the past three decades (King, 2000). However, the fact remains that milk production remains a significant limiting factor in determining piglet growth. With the emphasis on increasing litter size, high milk production is particularly important. Although research has provided more insight into the process of milk secretion, we have only a limited understanding of the physiological factors that

control the amount of milk a mammal produces. Previous work has suggested that the volume of milk produced is directly dependent upon the amount of lactose synthesized. There is evidence that suggests that milk volume is directly related to the expression of the  $\alpha$ -lactalbumin gene (Goodman and Schanbacher, 1991).

High milk production is vital for growth of the offspring. Low milk production is manifested not only by slow growth before weaning but also by slow growth later in life, since animal performance also suffers through the grower and finishing stages (Mahan and Lepine, 1991; Boyd and Kensinger, 1998; Miller et al., 1999). Current swine production management schemes attempt to maximize the number of piglets born per litter and piglet survival (Hartmann et al., 1984). In addition, pork producers have continuously reduced the duration of lactation to maximize the number of piglets born per sow per year. Currently, in the swine industry approximately 14-d lactation periods are common. In order to get maximum growth from larger litter sizes and shorter lactation, increased milk production in early lactation must be obtained. Early weaning has decreased neonatal mortality and increased litter sizes from selected high genetic merit sows but has also made milk production one of the most important limiting factors in piglet growth (Miller et al., 1999).

The effect of increased sow milk production on U.S. pork production is dramatic. Using current milk production values (Auldism et al., 1998), we estimate that increasing milk production by 10% would result in an additional \$2.46 per litter which would be worth \$28.4 million/year in the U.S. due to increased weight gains prior to weaning using a typical hog price of \$50/cwt. Modern sows are able to produce about 1 kg of milk per piglet per day for litter sizes up to 14 pigs (Auldism et al., 1998). This calculation does not consider the potential for decreased feed and labor costs, which would be associated with the higher postweaning weight gains and the shorter time to achieve market weight.

### Production of Transgenic Swine Expressing Bovine $\alpha$ -Lactalbumin

We have previously produced two lines of transgenic pigs containing the bovine  $\alpha$ -lactalbumin gene. This transgene has been inherited in a normal Mendelian fashion in  $F_1$  crosses. These animals have been extensively characterized and reported elsewhere (Bleck et al., 1996, 1998; Noble et al., 2002).

The bovine  $\alpha$ -lactalbumin gene was chosen for some very specific reasons. First, the expression of bovine  $\alpha$ -lactalbumin is the most tightly regulated and lactation specific of all the bovine milk protein genes (Goodman and Schanbacher, 1991; Mao et al., 1991). The unique expression of the bovine  $\alpha$ -lactalbumin gene makes its promoter and regulatory elements a useful mammary expression system in transgenic animals. In contrast to the caseins and  $\beta$ -lactoglobulin, the production of  $\alpha$ -lactalbumin mRNA and protein shows a dramatic rise

at parturition, remains elevated during lactation, and drops sharply during lactational cessation and involution. Next, the bovine, murine, and porcine  $\alpha$ -lactalbumin genes have all been sequenced and their proteins have molecular weights of about 14 kD (Brew and Grobler, 1992; Vilotte et al., 1992; Das Gupta et al., 1992). Thus the  $\alpha$ -lactalbumin gene and gene product have been quite well characterized, which was an important factor in its selection for our studies. Physiologically,  $\alpha$ -lactalbumin is produced at a concentration of approximately 0.2 to 1.8 mg/mL in the milk of most mammals, which makes it an excellent choice for a variety of species. Further, unlike  $\beta$ 1,4 galactosyltransferase,  $\alpha$ -lactalbumin is a mammary-specific protein whose expression is regulated by numerous hormones and growth factors (reviewed by Tucker, 1981; Forsyth, 1983; Kuhn, 1983; Vonderhaar, 1987; Ziska et al., 1988; Brew and Grobler, 1992). Among the proteins found in milk, it is unique in that its expression is tightly coupled to the onset of lactation after the gland is fully differentiated, suggesting that the regulation of expression of the  $\alpha$ -lactalbumin gene is fundamentally distinct from that of other milk proteins (Goodman and Schanbacher, 1991). Most milk proteins (i.e., caseins,  $\beta$ -lactoglobulin) are found in the mammary glands of pregnant animals as soon as secretory cells begin to differentiate in early to mid-pregnancy.

Because  $\alpha$ -lactalbumin is tightly correlated to milk production, it may be limiting for lactose synthesis. Several studies have shown that reducing  $\alpha$ -lactalbumin reduces milk production. Rats fed low-protein diets produced less milk and lactose, but  $\beta$ 1,4 galactosyltransferase content of the glands remained constant (Grimble and Mansaray, 1987). The lactose synthase activity of the glands isolated from protein-limited rats was half that found in control rats. The addition of bovine  $\alpha$ -lactalbumin to the gland homogenates of the protein-limited animals stimulated lactose synthase activity by 60%, compared with 10% in control animals (Grimble and Mansaray, 1987). These data indicate that  $\alpha$ -lactalbumin can be a limiting component in the lactose synthase complex and may be involved in control of milk production. Further, we selected a form of bovine  $\alpha$ -lactalbumin gene containing a specific polymorphism. This polymorphism is a single base variation located 15 basepairs 3' of the bovine  $\alpha$ -lactalbumin transcription start point (Bleck and Bremel, 1993). This is referred to as the +15 polymorphism. This allele has previously been associated with increased milk production in Holstein cattle (Bleck and Bremel, 1993) and shown to increase milk volume in transgenic mice (Bleck and Bremel, 1994).

The integrated information above, point to  $\alpha$ -lactalbumin as being an ideal candidate for overexpression in the mammary gland to study milk production and composition. Additionally, to address the question of whether  $\alpha$ -lactalbumin is limiting for milk production most thoroughly, an in vivo approach must be used. The production of  $\alpha$ -lactalbumin-deficient mice has allowed

the lactose synthase complex to be analyzed under conditions when  $\alpha$ -lactalbumin is greatly reduced or totally removed from the mammary gland (Stinnakre et al., 1994). As expected, mice with one remaining copy of the gene produce milk with lower lactose levels and mice without the gene produce no lactose. The milk from these mice is also higher in total solids, caused by a reduction in water content due to the lack of lactose, the osmoregulator. However, all knockout experiments only show the effect of removing the protein and yield no information about whether the protein is actually rate-limiting in the normal state. This can only be tested directly by increasing expression of specific genes. One group has replaced mouse  $\alpha$ -lactalbumin with human  $\alpha$ -lactalbumin, which is expressed at higher levels (Stacey et al., 1995). They show results suggesting there may be an increase in milk production in mice producing human  $\alpha$ -lactalbumin. However, those studies used only four animals and were preliminary. To obtain a definitive answer in swine,  $\alpha$ -lactalbumin must be overexpressed in the mammary gland and more careful studies of the phenotype must be performed. Even though a number of  $\alpha$ -lactalbumin expressing transgenic animals have been produced, our studies are presently the only experiments examining lactose and milk production in transgenic swine overexpressing  $\alpha$ -lactalbumin.

## Results

Our results have shown that the bovine  $\alpha$ -lactalbumin protein was expressed in the milk of our transgenic but not our control full-sibling gilts (Bleck et al., 1998; and Noble et al., 2002). Further, we have shown that milk composition is altered in the transgenics as compared to control gilts (Noble et al., 2002). Mean lactose concentrations in milk from transgenic sows was greater ( $P < 0.01$ ) than that for control sows over the entire lactation period (Noble et al., 2002). There was no difference in mean protein concentrations in transgenic and control sows over the lactation period. However, the mean total solids concentrations were lower ( $P < 0.002$ ) in transgenic sows, up to 12 h postpartum but not thereafter, than control sows. This work (Noble et al., 2002) has also shown that milk production increased ( $P < 0.001$ ) an average of 0.98 kg/day during d 3 to 9 of lactation in transgenic sows compared with control sows and that piglet growth rate significantly increased ( $P < 0.05$ ) in the transgenic gilts compared with the control sows ( $192 \pm 4$  g/d and  $168 \pm 4$  g/d, respectively). Please refer to Bleck et al., 1998 and Noble et al., 2002 for the specific details of these studies.

One of the curious results from the Noble et al. (2002) study is the apparent discontinuity of the enhanced milk production by transgenic sows and the increased growth rate of piglets. In this study, we saw a continued increase in growth in the absence of increased milk production. Although we are continuing to study this, the most plausible explanation is that there are some

changes that occur early in lactation in the piglets that allow this continued growth enhancement. The other explanation is that there continues to be additional bovine  $\alpha$ -lactalbumin protein in the milk of these gilts and that provides additional amino acids for piglet growth. Similarly, the milk composition, data presented in Noble et al. (2002) showed significant differences in milk composition, which may enhance piglet growth without increased milk yield in later lactation. For example, this may be an effect of greater consumption of colostrum and transition by the neonate piglet causing enhanced protein deposition later on. There is growing evidence that the nutritional environment of the newborn metabolically imprints the animal for future growth and development (Burrin et al., 1994; Burrin et al., 1997; Lucas, 1998; Waterland and Garza 1999).

Another open question in the  $\alpha$ -lactalbumin study is whether  $\alpha$ -lactalbumin is indeed limiting. The results of this transgenic work lend themselves to addressing that unresolved issue. We are continuing to work on this question; however, a substantial amount of work still needs to be done to address these many interesting questions.

### Summary

Bovine  $\alpha$ -lactalbumin transgenic swine have been produced with the purpose of studying the role of this protein on milk production and lactation. We have observed enhanced lactation performance and consequently enhanced litter growth performance (Noble et al., 2002). Our results have demonstrated that the bovine  $\alpha$ -lactalbumin gene can be expressed in the pig and the protein can be secreted into milk. To date, the animals containing the bovine  $\alpha$ -lactalbumin transgene have shown no obvious abnormal phenotype. Both the transgenic and control animals grew at the same rate, reached puberty at similar ages, gestated and farrowed normally, lactated normally, and their litters grew at rates consistent with or faster than controls.

Efficient and optimal pork production is reliant upon the production of healthy, fast growing piglets. Milk production in the first few weeks after birth is critical to modern swine production (Boyd and Kensinger, 1998). Our recent studies have demonstrated that overexpression of  $\alpha$ -lactalbumin in first parity gilts during lactation enhances lactational performance and enhances preweaning piglet growth rates (Noble et al., 2002). This is an important example of the application of transgenic technology to a livestock species with the expected outcome of subsequent enhancement of growth characteristics of the young. The advent of transgenic animal technology nearly 20 yr ago carried with it promise for biomedical research, pharmaceutical production in animal systems, and enhancement of food animal production. This promise has been realized in many areas, with the exception of production of transgenic livestock with rigorously demonstrated superior production characteristics. We demonstrate for

the first time based upon extensive production data, that transgenic technology can be used to enhance production characteristics of a farm species (Noble et al., 2002).

### Implications

Transgenic technology is a powerful tool for improving the production characteristics of livestock. One important application is enhancement of the growth of offspring. The use of the bovine  $\alpha$ -lactalbumin gene promoter and regulatory regions has great potential for studying the basic biology of milk secretion as well as for many additional applications in agriculture and biomedicine.

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