# 14 Changing the Composition and Properties of Milk

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Since the advent of the molecular biology era in the early 1970s, biotechnology has held great promise for improving animal agriculture. Since 1982, genetic engineering has held the promise of being able to significantly improve animal agriculture, with the dairy industry being one of the first industries to see this promise. Since then, the dairy industry has watched as transgenic technology has been applied to express foreign proteins in the mammary gland for the pharmaceutical industry. However, transgenic technology can also be used to alter the functional and physical properties of milk resulting in a milk with novel manufacturing properties. Work over the last decade on expression systems and in model species, such as the mouse, has now set the stage for the application of transgenic technology directly to the dairy animal to change the nutritional, antimicrobial and functional properties of milk.

## Introduction

Approximately 30% of dietary protein consumed in the Western world is obtained from milk, with the majority of this milk being derived from cows (Hambraeus, 1982). However, milk derived from dairy cows, goats or sheep differs significantly from human milk, as shown in Table 14.1. The most notable differences in general are the lower overall protein content of human milk and the much higher proportion of whey protein to casein protein. Milk consists of six major proteins, four being $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-casein which are present in the milk of most mammals. The remaining proteins are found in the whey fraction and include α-lactalbumin, which is common to all milks, and β-lactoglobulin (ruminants), lactoferrin (primates) or whey acidic protein (WAP; rodents, rabbits, camelids). The relative abundance of the various protein constituents of cow's milk varies between

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**Table 14.1.** Comparison of fat, casein and whey content of milk from ruminants and man (data from Davies *et al*., 1983).

| Species | Casein (g $l^{-1}$) | Whey (g $l^{-1}$) | Fat (g $l^{-1}$) |
|---|---|---|---|
| Cow | 28 | 6 | 37 |
| Goat | 25 | 4 | 45 |
| Sheep | 46 | 9 | 74 |
| Human | 4 | 6 | 38 |

breeds and genotypes, with the general proportions being approximately 31.5% $\alpha_{s1}$-casein, 29.5% β-casein, 8.5% $\alpha_{s2}$-casein, 11% κ-casein, 10% β-lactoglobulin, 4% α-lactalbumin, and 5.5% serum proteins and immunoglobulins (Davies *et al*., 1983).

The high reliance on dairy protein has resulted in considerable research being directed towards understanding lactation, the functional properties of milk and the molecular biology of the milk-specific genes. With the identification of various alleles of the major ruminant milk protein genes, genotypic effects on milk yield, composition and functionality were characterized. For example, milk from cows homozygous for the B allele of β-lactoglobulin had a higher content of fat, total solids, casein and whey protein than milk from homozygous A cows, while the amount of β-lactoglobulin was decreased (McLean *et al*., 1984). Milk from κ-casein B cows has a shorter rennet clotting time and increased curd firmness (Scharr, 1984), and increased natural heat stability (McLean *et al*., 1987). The variation in composition and functionality associated with natural genetic variants of milk protein genes suggests that making directed changes in these properties should also be possible.

Thus, with the demonstration that a gene construct could be transferred and expressed in mammals (Palmiter *et al*., 1982) it was natural that researchers should begin to speculate on the types of genetic changes that might usefully be made to the milk protein system. In 1984, Tom Richardson suggested that genetic engineering techniques should be applied to milk protein genes to 'change systematically the primary structure of a protein and to correlate these changes with alterations in protein functionality', with the aim of studying the function of these altered proteins in a bacterial system. Over the next decade Richardson and colleagues followed this initial general suggestion with a number of specific proposals for altering the milk protein system *in vivo* with the overall aim of changing the functional properties of the milk protein system. For example they suggested that the addition of extra copies of the κ-casein gene, in order to overexpress κ-casein, could result in an increase in the thermal stability of casein aggregates in milk (Kang and Richardson, 1985). A more complex proposal was to use site-directed mutagenesis to change $isoleucine_{71}$ to phenalanine in a clone of $\alpha_{s1}$-casein prior to gene transfer. The presence in milk of 10–20% of the $\alpha_{s1}$-casein as the mutant form might increase proteolysis and thereby

promote the faster ripening of cheese (Jimenez-Flores and Richardson, 1988; Yom and Richardson, 1993). All in all, Richardson and his colleagues put forward specific possible alterations in the properties of milk that might be gained by overexpressing, deleting or adding back a mutated form of most of the major milk protein genes (Kang and Richardson, 1985; Jimenez-Flores and Richardson, 1988; Yom and Richardson, 1993). Following these initial suggestions by Richardson's group, a number of articles have been written discussing the possible alteration of milk by transgenesis (Bremel *et al*., 1989; Muysson and Verrinder-Gibbins, 1989; Clark, 1992).

There have been a number of recent reviews written describing the major milk protein genes from a variety of mammals and their possible uses in transgenic studies (e.g. Bawden *et al*., 1994; Maga and Murray, 1995; Clark, 1996). Promoter elements from the $\alpha_{s1}$-casein, β-casein, α-lactalbumin, β-lactoglobulin and WAP genes, from one or more species, have now been used to drive expression of a transgene, usually in the mammary gland of mice (Table 14.2). Mammary gland-directed transgenes have been expressed, in addition to mice, in sheep, pigs, goats, rabbits and cows.

To date, most of the work on targeting transgene expression to the mammary gland of an animal has focused either on studying promoter function and identifying the important and necessary regions of DNA required for expression, or has been directed at producing biologically important and active proteins (such as pharmaceuticals) in the milk of a transgenic animal with the intent of recovering the protein of interest from the milk (Wilmut *et al*., 1991; Mercier and Vilotte, 1993; Bawden *et al*., 1994). It is now also possible to use transgenic animals to alter directly the properties and composition of the milk itself by genetically adding a new, or altered, protein to the milk protein system to cause effects and not for recovery of the protein for other uses. More specifically, we have been studying the addition of human lysozyme or a modified bovine κ-casein to mouse milk in order to affect the functional and physical properties of the milk protein system and thereby alter the manufacturing applications of milk (Maga and Murray, 1995). Work in the mouse also has demonstrated that antisense or ribozyme transgene constructs can be used to decrease the production of a targeted protein in milk (L'Huillier *et al*., 1996; Sokol *et al*., 1998).

## Milk Gene Products and Functions

The average composition of bovine milk is 86% water, 5% lactose, 4.1% fat, 3.6% protein and 0.7% minerals with a pH of 6.6–6.7. Milk composition remains relatively constant with the exception of fat content, which varies depending upon the breed of cow, feed type and stage of lactation (Johnson, 1974). All the components of milk are secreted by the mammary gland during lactation, however approximately half of the fat secreted into milk is derived from the serum while the remaining half is synthesized *de*

**Table 14.2.** Mammary gland-specific transgenic animals.

| Promoter | To express | Animal | Reference |
|---|---|---|---|
| Mouse WAP | Human t-PA | Mice, goats | Gorden *et al*., 1987; Ebert *et al*., 1991 |
| | Human SOD | Mice, rabbits | Hansson *et al*., 1994; Stromqvist *et al*., 1997 |
| | Human protein C | Mice, pigs | Velander *et al*., 1992a,b; Drohan *et al*., 1994 |
| | Mouse WAP | Pigs, sheep | Wall *et al*., 1991, 1996 |
| | Human factor VIII | Pigs | Paleyanda *et al*., 1997 |
| | Bovine TAP | Mice | Yarus *et al*., 1996 |
| Rabbit WAP | Human $\alpha_1$-antitrypsin | Mice | Bischoff *et al*., 1992 |
| | Human growth hormone | Mice | Devinoy *et al*., 1994 |
| Bovine $\alpha$-lactalbumin | Bovine $\alpha$-lactalbumin | Mice | Vilotte *et al*., 1989; Soulier *et al*., 1992; Bleck and Bremel 1993 |
| | Ribozyme $\alpha$-lactalbumin | Mice | L'Huillier *et al*., 1996 |
| Goat $\alpha$-lactalbumin | Goat $\alpha$-lactalbumin | Mice | Soulier *et al*., 1992 |
| Sheep $\beta$-lactoglobulin | Sheep $\beta$-lactoglobulin | Mice | Simons *et al*., 1987; Shani *et al*., 1992 |
| | Human $\alpha_1$-antitrypsin | Mice, sheep | Archibald *et al*., 1990; Wright *et al*., 1991 |
| | Human serum albumin | Mice | Shani *et al*., 1992 |
| | Human SOD | Mice | Hansson *et al*., 1994 |
| Bovine $\beta$-lactoglobulin | Bovine $\beta$-lactoglobulin | Mice | Hyttinen *et al*., 1998; Gutierrez-Adan *et al*., 1999 |
| | Human erythroprotein | Mice, rabbits | Korhonen *et al*., 1997 |
| Goat $\beta$-lactoglobulin | Goat $\beta$-lactoglobulin | Mice | Ibanez *et al*., 1997 |
| Rat $\beta$-casein | Rat $\beta$-casein | Mice | Lee *et al*., 1988 |
| | Bacterial CAT | Mice | Lee *et al*., 1989 |
| Rabbit $\beta$-casein | Human interleukin-2 | Rabbits | Buhler *et al*., 1990 |
| Goat $\beta$-casein | Goat $\beta$-casein | Mice | Persuy *et al*., 1992; Roberts *et al*., 1992 |
| | CFTR | Mice | DiTullio *et al*., 1992 |
| | Bovine $\kappa$-casein | Mice | Gutierrez *et al*., 1995 |
| | Human t-PA | Goats | Ebert *et al*., 1994 |
| Bovine $\alpha_{s1}$-casein | Bovine $\alpha_{s1}$-casein | Mice | Clarke *et al*., 1994 |
| | Bovine $\alpha_{s1}$-casein | Mice | Rijnkels *et al*., 1998 |
| | Bacterial CAT | Mice | Clarke *et al*., 1994 |
| | Antisense CAT | Mice | Sokol *et al*., 1998 |
| | Human GM-CSF | Mice | Uusi-Oukari *et al*., 1997 |
| | Human IGF-1 | Rabbits | Brem *et al*., 1994 |
| | Human urokinase | Mice | Meade *et al*., 1990 |
| | Human lysozyme | Mice | Maga *et al*., 1994 |
| | Human lactoferrin | Mice, cow | Krimpenfort *et al*., 1991; Platenburg *et al*., 1994 |

*novo* in the mammary gland. The six major mammary gland gene products, $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-casein, β-lactoglobulin and α-lactalbumin, account for the 3.6% or 30–35 g $l^{-1}$ of protein present in cows' milk. The caseins are present at a ratio of $\alpha_{s1}$:$\alpha_{s2}$:β:κ at 3:1:3:1. The caseins contribute the majority of the protein, 80% or 24–28 g $l^{-1}$, while the whey proteins account for 20% of the total protein or 5–7 g $l^{-1}$. A typical cow yields 10,000 pounds of milk over a 305-day lactation period.

## Caseins

The caseins are single polypeptide chains that are random in structure with little secondary structure, high average hydrophobicity and a net negative charge. Caseins contain many essential amino acids but are low in cystine content (Fox, 1982). Caseins are post-translationally phosphorylated, essential for calcium binding, at accessible serine residues in the amino acid sequence Ser/Thr-X-Glu/Ser-$PO_4$, where X is any amino acid (Mercier, 1981).

The caseins are present in milk in the form of micelles, or a colloidal suspension of proteins of the order of 20–600 nm in size (Fox, 1982). $\alpha_{s1}$-, $\alpha_{s2}$- and β-casein form the core of the micelle while the amphiphilic κ-casein lies on the surface. Due to their unordered and highly hydrophobic nature, the caseins can aggregate with themselves and the other caseins by hydrophobic interactions, hydrogen bonding, electrostatic attractions (Ca to Ser-$PO_4$) or repulsions (Ser-$PO_4$ to Ser-$PO_4$). $\alpha_{s1}$- and β-casein tend to self associate while $\alpha_{s1}$- and κ-casein form aggregates with each other.

Micelles function to sequester and transport calcium in a usable form to the newborn for bone development. The structure of the micelle can be disrupted by acid or the enzyme rennin. Acid acts to neutralize the negative charges of the phosphate and promote association leading to isoelectric coagulation of the caseins when the pH reaches 4.6. This happens in the stomach as the acid pH of the stomach causes casein precipitation, which makes the milk protein more digestible. Rennin is a combination of chymosin and pepsin isolated from calf stomach, which specifically cleaves κ-casein at the Phe105–Met106 peptide bond. Once cleaved, para κ-casein, or the hydrophobic N-terminal of the protein, stays associated with the micelle and the hydrophilic C-terminal is released from the micelle. Cleavage of κ-casein causes destabilization of the micelle structure resulting in precipitation of the caseins, thus separating the milk into two distinct fractions, the non-soluble caseins and the soluble whey proteins.

## Whey proteins

Whey proteins are usually compact and globular with a relatively uniform distribution of polar, non-polar and charged residues in contrast with the

clustering of similar residues that characterize the caseins (Fox, 1982). The whey proteins tend to be high in cysteine content, whose disulphide bonds can contribute to flavour. Whey proteins are more hydrophilic than the caseins and bind more water. The whey proteins are so named due to the fact that they stay in solution after the caseins are precipitated by acid or rennet.

## Transgene Expression in the Mammary Gland

Representative alleles of the major milk genes ($\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-casein, α-lactalbumin, β-lactoglobulin, and whey acidic protein) have been cloned from various species and the promoter regions have been isolated and characterized (Table 14.2; for review see Bawden *et al.*, 1994; Maga and Murray, 1995). The promoters from most of these genes have been used to generate transgenic animals, usually mice, but also cattle, sheep, pigs and goats, which produce foreign proteins in the mammary gland (Table 14.2; Maga and Murray, 1995). The sheep β-lactoglobulin, goat β-casein and bovine $\alpha_{s1}$-casein promoters have been the most efficient at supporting good levels of heterologous protein expression in the mammary gland of transgenic mice (Simons *et al.*, 1987; Meade *et al.*, 1990; Persuy *et al.*, 1992). Better expression is obtained in most cases if genomic DNA sequences, rather than cDNA, are used and the incorporation of untranslated exons and introns may contribute to increased expression of the transgene (Whitelaw *et al.*, 1991).

There are a number of conclusions that can be drawn from the studies to date on expressing transgenes in the mammary gland. First, all of the mammary gland-specific promoters can be used to direct tissue and developmentally correct expression of a transgene to the mammary gland. However, expression is obtained with varying degrees of efficiency that suggests there may be one or more as yet unidentified *cis*-acting control sequences associated with these genes. There are some species differences with respect to how specific promoters will function, e.g. the WAP promoter is tissue specific in pigs but is not in sheep (Wall *et al.*, 1996). Second, the expression of a foreign protein in the mammary gland, with secretion into the milk, in most cases does not appear to affect the functioning of the mammary gland. Exceptions include impaired lactation of sows from three different transgenic lines expressing mouse WAP in the mammary gland (Shamay *et al.*, 1992) and the work of Bleck *et al.* (1995) showing abnormal lactation in mice expressing bovine β-casein. Third, mammary gland-directed transgenes can be successfully inserted and expressed in all species of mammals thus far studied, including mice, rabbits, cattle, sheep, goats and pigs (Table 14.2).

## Transgenic Ruminants

A number of laboratories worldwide have successfully produced transgenic sheep (Hammer *et al.*, 1985; Simons *et al.*, 1988; Murray *et al.*, 1989;

Bawden *et al.*, 1995; Schnieke *et al.*, 1997), goats (Ebert *et al.*, 1991) and cattle (Krimpenfort *et al.*, 1991; Hyttinen *et al.*, 1994; Cibelli *et al.*, 1998), although the efficiency of producing transgenic ruminants remains low. All of the research to date with transgene expression directed towards the mammary gland in ruminants has focused on the production of pharmaceutical proteins, such as $\alpha_1$-antitrypsin in sheep (Wright *et al.*, 1991), tissue plasminogen activator in goats (Ebert *et al.*, 1991), and human lactoferrin in dairy cattle (Krimpenfort *et al.*, 1991) or testing the efficacy of specific heterologous promoters to reliably direct transgene expression in the mammary gland (Wall *et al.*, 1996). Recent advances in cloning sheep (Schnieke *et al.*, 1997; Wells *et al.*, 1997; Wilmut *et al.*, 1997) may result in increased efficiencies in the production of transgenic ruminants as well as allowing for targeted insertion of the transgene sequences. Nevertheless, with sufficient resources it is now possible to reliably produce transgenics in all of these species and obtain tissue and developmentally correct expression of the transgene in the mammary gland.

## What Might We Do to Alter the Properties of Milk?

As mentioned in the introduction, a number of papers have been written over the last 14 years discussing the possible alterations that could usefully be made by genetic engineering to improve the value of milk as an agricultural commodity (e.g. Richardson, 1984; Kang and Richardson, 1985; Jimenez-Flores and Richardson, 1988; Bremel *et al.*, 1989; Muysson and Verrinder-Gibbins, 1989; Oh and Richardson, 1991; Clark, 1992, 1996; Yom and Richardson, 1993; Bawden *et al.*, 1994). Box 14.1 lists five basic types of changes in milk that might be usefully considered. Within these broad areas, a wide variety of modifications to milk have been suggested, including: adding extra copies of an existing gene ($\alpha_{s1}$-, κ- and β-casein), down-regulating the expression of a gene (α-lactalbumin), adding new genes (human lysozyme or lactoferrin), removal of a gene (β-casein, β-lactoglobulin or acetyl-CoA carboxylase), and adding a mutated gene ($\alpha_{s1}$-, κ- and β-casein). Preliminary research has been carried out using transgenic mice as model systems in the first four of these categories. Given the present state of transgenic technology in ruminants, all of these modifications, and others not listed, could now potentially be made.

**Box 14.1.** Areas where milk might be usefully manipulated.

- Altering the proteins to change the manufacturing properties of milk.
- Increasing the antimicrobial activity of milk.
- Altering the type and amount of fatty acids in milk.
- Changing the amino acid composition of milk to improve human nutrition.
- Increasing the overall protein content of milk.

## What We Have Learned from Transgenic Mouse Models

### Adding extra copies of an existing gene

The addition of more κ-casein to the milk protein system could affect the physical properties of the milk since κ-casein is directly involved with micelle formation, structure and size (Waugh, 1971; Fox, 1982; Schmidt, 1982). An increase in κ-casein could increase the thermal stability of casein aggregates (Jimenez-Flores and Richardson, 1988) and act to decrease micelle size (Fox, 1982). A smaller micelle diameter would lead to a larger available surface area, which would result in a more consistent and firmer curd as well as an increase in cheese yield. These modified properties of milk could be of great benefit and interest to the dairy industry. Results on milk from mice expressing the bovine κ-casein gene showed significantly greater rennet gel strength and decreased micelle particle size when compared with milk from non-transgenic, full-sib control mice (Gutiérrez-Adán *et al.*, 1996).

### Down-regulating the expression of a gene

The expression of a gene can be down regulated *in vivo* in a developmentally and tissue-specific manner using transgenes expressing antisense or ribozyme messages (for review see Sokol and Murray, 1996). Two experiments have been completed assessing the efficacy of using antisense or ribozyme constructs to inhibit mRNA translation in the mammary gland. In both cases the target was message-derived from the expression of a non-endogenous transgene. In the first case, L'Huillier *et al.* (1996) used a ribozyme construct targeting the mRNA of bovine α-lactalbumin. Using three different lines of transgenic ribozyme mice crossed to bovine α-lactalbumin transgenic mice, these authors were able to show a 50–78% reduction in the level of bovine α-lactalbumin protein in the milk of double hemizygous females. There was no effect of the bovine-based α-lactalbumin ribozyme on the translation of the endogenous mouse α-lactalbumin mRNA. Sokol *et al.* (1998), using the same double hemizygous strategy, evaluated the ability of antisense and antisense/ribozyme constructs to down-regulate the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene in the mammary gland of transgenic mice. In this case, antisense and antisense/ribozyme constructs were equally efficient, leading to approximately an 80% reduction in the amount of CAT protein secreted into the milk.

### Adding new genes

Human lysozyme transgenic mice were studied to determine the consequences on some basic rheological and antimicrobial properties of

milk as a result of having human lysozyme expressed in the milk. Lysozymes are ubiquitous enzymes found in avian egg whites and mammalian secretions such as tears, saliva and milk that are positively charged at physiological pH (Jolles and Jolles, 1984) and have an inherent antimicrobial activity (Phillips, 1966). If human lysozyme was present in bovine milk at a significant level, two main effects could be expected. First, because of its antimicrobial activity, lysozyme may reduce the overall level of bacteria in milk thus decreasing disease in the udder and overall bacterial levels in the milk. As lysozyme is considered to be part of passive immunity and the natural defence against bacteria, viruses, parasites and fungi in human milk (Chang, 1990), there may be human health advantages as well. Second, due to the net positive charge, lysozyme may be able to interact with the negatively charged caseins to produce a milk with altered functional and physical properties. In studies using transgenic mice expressing human lysozyme in their milk at an average concentration of 0.38 mg $ml^{-1}$ the rennet clotting time of the milk was decreased by 35%, gel strength of rennet induced gels was significantly higher in milk from the transgenic mice than in milk from control mice, while the average size of the micelles tended to be smaller (Maga *et al.*, 1995). Milk from these same transgenic lines was found to be bacteriostatic against two cold spoilage organisms, *Pseudomonas fragi* and *Lactobacillus viscous*, and a mastitis-causing isolate of *Staphylococcus aureus* (Maga *et al.*, 1998).

### Removal of a gene

In order to determine the consequences on the milk protein system of deleting a major milk protein, Kumar *et al.* (1994) produced a β-casein knockout line of mice. Mice heterozygous for the disruption of this gene had reduced levels of β-casein in the milk, while animals homozygous for the knocked-out allele lacked β-casein in the milk. In the homozygous knockout animals the overall protein concentration was reduced, although there was an increase in the amount of the other milk proteins. The homozygous knockout females lactated normally, with the milk having correctly assembled micelles, although the diameter of the micelles was reduced. Kumar *et al.* (1994) concluded that β-casein is a non-essential component of the milk protein system, thus illustrating that profound changes can be made in the composition of milk without disrupting the general organization of the micellular system.

## Conclusions

Throughout this review we have tried to use examples to illustrate that the science has progressed sufficiently over the last 14 years for us to be

optimistic that transgenic technology can, and will, be successfully used to improve the dairy animal. We have not tried to cite every possible paper and apologize if a particularly important study or suggestion has been missed. However, the papers cited do illustrate that we can now construct a transgene that will have a good chance of functioning in the mammary gland of a transgenic female at a high level in a tissue- and developmentally appropriate manner. Transgenic sheep, goats and cattle can now be routinely made, although the efficiency is still quite low and the costs high, particularly for cattle.

The advent of cloning may increase our ability to produce transgenic ruminants on two fronts. First, as the transgene is inserted during the cell culture phase, each offspring born will be transgenic and each one will have the same insertion point. The fact that they are clones is only relevant to the extent that care must be taken to avoid inbreeding. Secondly, the ability to rederive animals from cells in culture finally opens the possibility of doing either gene knockout or gene replacement experiments, thus increasing the options available for altering the composition of milk and the control of lactation. The ability to carry out the full range of genetic modifications routinely produced in mice is now available for use in dairy animals, although much research remains to be done with respect to identifying appropriate target phenotypes for alteration, the construction of the required transgenes, and on improving the efficiency of production of transgenic livestock.

Experiments performed in mice have shown that the mammary gland and milk systems are robust and can be altered and added to using a wide variety of different proteins and still function. Furthermore, the system can be altered such that predictable changes in the functional and antimicrobial nature of milk can be produced. The results in mice suggest that human lysozyme and κ-casein are good candidates for altering properties of milk in a beneficial fashion and demonstrate that transgenic technology can be used for agricultural purposes as well as for studying gene function and the production and recovery of novel proteins in the milk of transgenic livestock. However, here at the beginning there is no lack of good ideas for possible changes to the mammary gland and the milk protein system: one only has to start with the papers of Tom Richardson.

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