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Improving milk for human consumption through genetic engineering technologies

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ABSTRACT

Improved living conditions, food security and particularly access to comprehensive health-care systems have resulted in a continuous increase of the human life expectancy. However, living longer does not immediately mean that quality of life can be maintained into old age, which is commonly compromised by disease. The full benefit of a longer life can only be realised when the later stages of life can be enjoyed in good health. This has generated strong demand for new innovative foods that are not only safe and nutritious but also have health enhancing properties. Genetic modification technology provides a direct approach to enhance existing attributes that are beneficial for human health, minimize any undesirable characteristics or enable the introduction of novel, health promoting traits. Focused on milk as an important human food source, we will review the humble beginnings of testing transgenic approaches with mouse models, transfer of these simple overexpression strategies into livestock species, application of programmable nucleases for the targeted modifications of milk characteristics and discuss future opportunities that are becoming feasible with today's sophisticated technologies.

INTRODUCTION

Milk is a complex biological fluid that evolved to provide a single source of balanced nutrition able to support the growth and survival of all mammalian offspring. Because of its high nutritional value, humans have embraced milk of dairy animals as a source of high

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quality nutrition with health promoting characteristics. Dairy milk is consumed not only in its raw form but is also processed into many different dairy foods, including yoghurt and cheese. However, nature has optimised dairy milk for the nutritional demands of calves, lambs and kids and hence, is not specifically tailored to provide the best possible benefits for human consumption. The advent of transgenic technology with the ability to make targeted changes to the composition of milk made it feasible to improve dairy milk for human consumption. It immediately triggered intensive discussions on possible strategies to improve the overall composition of milk, the fatty acid composition of milk fat as well as individual milk proteins (Boland *et al.* 1992; Jimenez-Flores and Richardson 1988; Wilmut *et al.* 1990). Since then, the technical capabilities for the efficient and precise modification of livestock genomes have advanced tremendously (Laible *et al.* 2015; Tan *et al.* 2016). In the following we will provide a brief summary of past and current efforts and what may be possible in the near future.

TRANSGENIC MOUSE MODELS WITH IMPROVED MILK CHARACTERISTICS

Initially these concepts could only be tested in mouse models which showed the feasibility of overexpressing the major milk proteins from livestock species (Wall *et al.* 1997). Correct incorporation of recombinant livestock caseins into the casein micelles of mouse milk further validated this approach (Gutierrez-Adan *et al.* 1996; Hiripi *et al.* 2000; Hitchin *et al.* 1996; Persuy *et al.* 1995). Compared to cows' milk, human milk contains much higher amounts of the two anti-microbial whey proteins: lysozyme and lactoferrin. Overexpression of the human forms of lysozyme (Maga *et al.* 1998) and lactoferrin (Seyfert *et al.* 1996) was tested in mouse models to demonstrate the feasibility of humanizing the milk of another species to provide additional health benefits by boosting passive immunity following consumption. In addition, different strategies were investigated to address lactose-intolerance. This affects more than 70% of adult consumers of cows' milk and causes intestinal disorders due to inadequate intestinal lactase activity levels resulting in insufficient lactose digestion after the consumption of milk (Sahi 1994). As alternatives to expensive post-harvest milk processing two strategies for reducing the milk lactose content were evaluated in transgenic mouse models. Disruption of the gene encoding α-lactalbumin, which is an essential component of the lactose synthetase complex, resulted in lactose-free milk (Stinnakre *et al.* 1994). But because lactose is also the main osmotic regulator in milk, a

lack of lactose resulted in highly viscous milk and inability of the transgenic mice to sustain lactation and adequate nutrition for the suckling young. To dissect the impacts of osmolarity and lactose levels Jost and co-workers overexpressed a mammalian lactose hydrolysing enzyme in the mammary gland (Jost *et al.* 1999). With this approach, milk lactose levels were reduced by 50-85% but because lactose was converted into the osmotically active monosaccharides, glucose and galactose, this did not interfere with the osmolarity of the milk.

In addition to proteins and carbohydrates, milk fat is an important nutritional component of milk that has significant impact on human health. Like other animal fats, milk fat has a high content of saturated fatty acids which are considered 'unhealthy' and have been associated with cardiovascular and coronary heart disease. Hence, reducing unhealthy, saturated fats in favour of healthier unsaturated fats is a major target for the improvement of dairy products. A promising concept was the complementation of transgenic mice with a desaturase, normally not found in vertebrates, which would enable the transgenic mice to endogenously synthesise essential poly-unsaturated fatty acids. Indeed, conversion of dietary derived n-6 fatty acids into omega-3 poly-unsaturated fatty acids, known for lowering the risk of morbidity and mortality from atherosclerosis and coronary heart disease, could be achieved with the expression of the n-3 fatty acid desaturase from *Caenorhabditis elegans* following the insertion of the fat-1 gene (Kang *et al.* 2004; Kao *et al.* 2006).

TRANSFER OF EARLY CONCEPTS FROM MOUSE TO LIVESTOCK

The early mouse models can only be regarded as feasibility studies that validated the principle concepts for changing individual milk components. Because the composition of milk, and with it the closely linked functional characteristics of milk, varies greatly between different species, the true effects on changes to the composition of the milk, any associated nutritional and health benefits and potential impact on the processing characteristics of the milk resulting from any direct changes to milk components ought to be assessed in dairy animals as the ultimate target species for producing dairy foods for human consumption.

The concept of overexpressing a milk protein was first transferred to livestock for an agricultural application with the aim of improving the nutrition and survival of piglets.

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Transgenic sows that overexpressed bovine α-lactalbumin in their milk could markedly improve the survival rates of suckling piglets and hence increases the overall efficiency of pig production (Wheeler *et al.* 2001). In an attempt to improve milk for human consumption, the human variant of α-lactalbumin was overexpressed in cows' milk (Wang *et al.* 2008). Analyses of the milk showed no apparent changes to the abundance of endogenous milk proteins when compared to milk produced by wild-type cows with nutritional benefits expected from the additional human α-lactalbumin in the milk. Casein represents 80 % of the protein content of milk and essentially constitutes the milk component that makes up cheese when milk is processed into cheese. Casein's nutritional importance and relevance for the processing of milk makes it a highly valuable milk component. To increase casein content, cheese yield and improve milk processing properties, bovine β- and κ-casein were overexpressed in transgenic cattle (Brophy *et al.* 2003). This resulted in a threefold increase in κ-casein whilst β-casein and total protein levels were only modestly elevated. The increased levels for these two caseins were associated with complex changes in the abundance of endogenous milk proteins, milk fat content and composition and mineral balance (Laible *et al.* 2016). The enhanced content of sialic acid, considered to be an important nutrient for the developing brain and the augmented levels of beneficial micronutrients such as calcium, magnesium and zinc underlines the milk's potential for improved health characteristics. The impact of the changed milk composition on the physicochemical properties, important for dairy processing, was immediately noticeable with a marked difference in the colour of the milk produced by the transgenic cows compared with wild-type, and by the substantially reduced size of the main protein particles in milk, the casein micelles (Laible *et al.* 2016; Laible *et al.* 2007). To determine whether these changes may have a positive effect on different milk processing processes needs further investigation. Other pioneering studies were aimed at enhancing the abundance of milk proteins with anti-microbial activity. Overexpression of the human forms of lysozyme and lactoferrin in goats and cattle further improved the health benefits of dairy milk by boosting innate defence mechanisms against pathogenic microorganisms (Cooper *et al.* 2015). A longer shelf life of the milk and protection of lactating animals against mastitis-causing pathogens as a consequence of increased levels of these anti-microbial proteins in milk provide further evidence for the milk's health-promoting effects (Maga *et al.* 2006; Simojoki *et al.* 2010).

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The concept of engineering a dairy animal for the production of healthier milk fat was first explored with transgenic goats. The mammary gland-specific overexpression of the key enzyme involved in converting saturated fatty acids into mono-unsaturated fatty acids, stearoyl-CoA desaturase, improved the milk fat composition by increasing the levels of unsaturated fatty acids and lowering the amounts of saturated fatty acids (Reh *et al.* 2004). However, the transgenic goats were unable to sustain the altered fatty acid ratios possibly due to instabilities of the transgene-derived mRNA. Enabling livestock with the endogenous production of omega-3 fatty acids, which are highly beneficial for human health and mainly available through the consumption of oily fish, provides a new, more sustainable food source for omega-3 fatty acids and was demonstrated in pigs, sheep and cattle (Lai *et al.* 2006; Saeki *et al.* 2004; Zhang *et al.* 2013; Cheng *et al.* 2015). Transgenic cattle, constitutively expressing the *Caenorhabditis elegans* fat1 gene that was optimised for mammalian expression, produced milk with high levels of omega-3 poly-unsaturated fatty acids and strongly reduced levels of the n-6 fatty acids (Wu *et al.* 2012).

For many years, technical limitations restricted the modification of livestock genomes to the overexpression of transgenes that were integrated into random chromosomal sites (Laible *et al.* 2015). Although still based on a simple overexpression strategy, the expression of small interfering RNAs to degrade or block specific mRNAs offers a relatively simple way to modulate the activity of an endogenous gene. The concept was verified in cattle with the expression of artificial microRNAs designed to target the mRNA for the bovine milk protein gene for β-lactoglobulin (Jabed *et al.* 2012). Milk produced by a cow expressing the microRNAs under the control of a milk protein promoter no longer contained detectable levels of β-lactoglobulin, a major allergen in cows' milk.

ENGINEERING MILK PHENOTYPES WITH PROGRAMMABLE NUCLEASES

The recent development of programmable nucleases has lifted most of the technical limitations and enabled the efficient, site-specific modification of livestock genomes (Tan *et al.* 2016). These nucleases, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered, regularly interspaced short palindromic repeat

(CRISPR) – CRISPR-associated nuclease 9 (Cas9), can be designed to target a unique site in the genome where they introduce a DNA double strand break. This induces the error-prone non-homologous end joining (NHEJ) repair mechanism or, in the presence of a homologous repair template, can facilitate homology-directed repair (HDR). Repair of DNA double strand breaks by NHEJ frequently generates small deletions and insertions (indels), which can be utilised to disrupt genes, while HDR can be employed to modify a genomic locus with full precision, including insertions of specific mutations or knock-ins of transgenes. To reduce the allergenicity of cows' milk, ZFN-induced NHEJ was applied to disrupt the gene for β-lactoglobulin. The live calf that was produced was biallelically modified at the target site. However, both introduced mutations were small in-frame deletions still permitting the production of a truncated form of β-lactoglobulin, potentially still retaining the allergenic properties (Yu *et al.* 2011). The use of a HDR-based strategy ensures a more predictable outcome with the insertion of a defined mutation. This approach was recently verified by the TALEN-mediated introduction of a pre-mature stop codon at the beginning of the gene for β-lactoglobulin directly in bovine embryos (Wei *et al.* 2015). The efficient knock-in capabilities through the application of programmable nucleases were also used to humanise goats' milk and improve its nutritional characteristics. The human milk protein genes for lactoferrin and α-lactalbumin were knocked-in to endogenous milk protein genes in goats essentially replacing the endogenous milk protein β-lactoglobulin with a different human whey protein (Cui *et al.* 2015; Zhu *et al.* 2016). In addition, cattle were generated with a knock-in of the human lysozyme gene into the bovine β-casein locus albeit for providing protection for the lactating cow against infections of the mammary gland rather than primarily for nutritional reasons (Liu *et al.* 2014).

NEW OPPORTUNITIES GENERATED BY TECHNOLOGICAL ADVANCEMENTS

Genome editing with programmable nucleases makes it possible to very efficiently introduce natural mutations that have been shown to be causatively linked to specific phenotypes. This was recently exemplified with the introduction of the POLLED mutation known from beef cattle into dairy cattle (Carlson *et al.* 2016). Importantly, genome editing leaves no technological footprint, rendering the edited mutation indistinguishable from a naturally occurring mutation. Based on rapidly growing sequence information on individual dairy cattle and associated phenotypic data sets, genome-wide association screens are used

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to identify sequence variants, or in other words mutations, that are causatively linked with a specific trait. Because milk production and composition are important traits with economic relevance, tremendous efforts go into the discovery of causative traits with relevance for milk. At present, only a small number of causative mutations with impact on milk have been identified. One such already characterised mutation is a lysine to alanine substitution in the DGAT1 gene which results in increase milk fat content (Grisart *et al.* 2004; Spelman *et al.* 2002). However, it can be expected that this number will rapidly increase in the coming years providing many new candidates for the improvement of milk. Once causative mutations are known, they can be directly introgressed by genome editing into elite genetic backgrounds of high performing dairy cattle, fast-tracking the efficient production of milk with improved characteristics. Undesirable characteristics, such as allergenicity can be addressed by disrupting the relevant gene function which was exemplified for β-lactoglobulin (Wei *et al.* 2015). If the immune-reactive regions of the proteins have been identified, it would even be possible to just delete the encoding sequence for the allergenic epitope to minimise any changes to the overall composition of the milk. HDR with programmable nucleases provides now also an efficient method to humanise milk by replacing the endogenous milk protein gene variants of dairy animals with the corresponding human gene variants which might be better suited for human consumption. An alternative approach which does no longer rely on naturally evolved variants could involve the generation of entirely novel variants. Milk proteins could be improved through rational design with the aim of increasing the nutritional value and digestibility of the protein component in milk or enhance processability of milk into various dairy products. Milk proteins are also a source of bioactive peptides that are released during digestion and could be engineered to increase the availability of these peptides which could then be modified to serve as vaccines (Whitelaw *et al.* 2016). Similarly, specific nutritional requirements could be addressed by modifying milk proteins to optimise their content of essential amino acids or tailor amino-acid contents according to specific dietary conditions such as low phenylalanine for sufferers of phenylketonuria who cannot metabolise the amino acid (Laible 2009). Thus, this technology offers a unique opportunity to address individual dietary needs and provides a direct route towards personalised nutrition.

CONCLUSION

Transformational improvements of the technical abilities to genetically modify livestock with precision and efficiency makes it now feasible to better customise dairy milk for human consumption. However, technological interference with animal derived foods through genetic modification has been tarnished by low public acceptance. Overwhelming scientific evidence supports the view that the technology carries no more risk than other commonly practised food production technologies (Anon 2010). It will be up to the scientists to provide compelling evidence of the safety of any new products to consumers and to the welfare of production animals and, more importantly, to invent novel products that offer very clear consumer benefits to change today's predominant consumer perception. However, for consumers to have the choice they deserve and the opportunity to decide for themselves, these products would need to be available for purchase. This will only be possible when the present regulatory uncertainties are replaced with a predicable regulatory approval pathway to market.

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