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Genetically engineering milk

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It has been thirty years since the first genetically engineered animal with altered milk composition was reported. During the intervening years, the world population has increased from 5bn to 7bn people. An increasing demand for protein in the human diet has followed this population expansion, putting huge stress on the food supply chain. Many solutions to the grand challenge of food security for all have been proposed and are currently under investigation and study. Amongst these, genetics still has an important role to play, aiming to continually enable the selection of livestock with enhanced traits. Part of the geneticist's tool box is the technology of genetic engineering. In this Invited Review, we indicate that this technology has come a long way, we focus on the genetic engineering of dairy animals and we argue that the new strategies for precision breeding demand proper evaluation as to how they could contribute to the essential increases in agricultural productivity our society must achieve.

Keywords: Genetic engineering, genome editing, milk, food security, review.

What is milk

Milk is part of the definition of what a mammal is. It is one of the major protein sources on the planet. Seen from the perspective of the mammalian neonate, milk is the fuel required for growth and survival. This in itself is enough to make milk perhaps the most important food on the planet, but milk is more than just something, albeit a very important something, that neonates must have. For the dairy farmer, it is his primary product ensuring his livelihood, while the food producer recognises its chemical properties as the basis for a wide range of commercially valuable food products.

Milk as a commercial food product is produced all around the world with one-third of the global production residing in India, USA and China. There is no country that does not have milk and milk products as part of their economy. For some countries, for example New Zealand, it is a major component of the annual commodities exports. We benefit from a number of domesticated dairy species – cow, buffalo, goat, sheep camel and yak – with cow's milk alone amounting to over 700 m tonnes in 2013 with more than 6bn people consuming dairy products (FAO, 2013).

Although milk is the result of evolution over considerable periods of time, one can still ask whether commercially available milks and milk products are optimal for current societal needs. Many around the world cannot consume milk or dairy products due to allergies or lactose intolerance. Dairy herds in the diverse geographical regions of the world show huge differences in milk production volumes. In highly productive dairy herds, we are challenged to achieve simultaneous genetic gain in milk production and reproductive performance while optimising animal health, reducing the impact of disease and enhancing welfare standards. If the range of proteins found in milk could be expanded, alternative uses of milk could be envisaged. There are many ways in which milk could be enhanced. We describe one route to achieve this goal, that of using genetic engineering technology. We focus on the mammary gland and milk, and hence food security, whilst being aware that manipulations of other aspects of the physiology and pathophysiology of the lactating animal could have beneficial impacts on its health and welfare.

Milk has been a target for the genetic engineer since the emergence of this technology 30 years ago (Simons et al. 1987). From the early days of this research field, the majority of effort has been to develop livestock as animal bioreactors of biomedical proteins (Clark et al. 1989). This goal is still actively pursued and has been the focus of numerous reviews (Wilmut & Whitelaw, 1994; Lubon et al. 1996; Houdebine, 2000; Kind & Schnieke, 2008; Cooper et al. 2015). Given the advent of new tools to engineer the genome of livestock we believe the opportunity to engineer milk proteins is likely to re-emerge.

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Brief history of GM milk

The first genetically engineered mammals were produced at the end of the 1970s (Palmiter & Brinster, 1986; Fig. 1). Those working in large animal biology quickly saw the potential for agriculture and embarked on two complementary avenues of research. The first attempted to modify growth through growth hormone-encoding transgenes. Although extremely useful in fuelling the early progress of the field, and providing for much ethical debate, this research direction was not initially successful, with the 'Beltsville' pigs displaying a range of undesirable phenotypes (Pursel et al. 1989, 1990). Some have sustained research activity in this area of research, with minor successes in changing back fat being achieved along the way (Pursel et al. 2004). Recently, the goal of altering meat composition has seen a resurgence of activity, largely revolving around the myostatin gene (or growth derived factor 8) with a number of groups around the world actively engaged in projects aiming to alter farm livestock muscle growth (Crispo et al. 2015; Proudfoot et al. 2015; Qian et al. 2015).

The alternative strategy, that of modifying milk composition, kick started with mice that expressed the whey protein β-lactoglobulin (Simons et al. 1987). Mice do not normally produce this protein. This study definitely demonstrated that substantial changes to milk composition could be engineered without causing deleterious phenotypic effects. This was a pointer for what is still an active area of academic research which has also progressed to the commercial sector and human clinic: farm livestock could be used as 'bioreactors' for desirable biomedical proteins. Using the gene promoters from various milk protein genes, a number of human proteins have been made in the mammary gland of transgenic livestock. Much has been done to optimise harvest and purification of these proteins e.g. (Zhao et al. 2015), with the first to successfully navigate through the appropriate regulatory process and reach the clinic in 2006 (Pollock et al. 1999; Anon 2009). In parallel, a diverse range of transgene designs were evaluated providing valuable information for the entire transgenic community.

The research activity involved in progressing farm livestock as animal bioreactors of proteins had a profound impact on the field of genetically engineered livestock. This was initially achieved through the use of pronuclear injection technology which involved the direct injection of the transgene DNA into the zygote (Hammer et al. 1985). This method enables transgenes to be added to the genome. Pronuclear injection is a robust but inefficient method, this inefficiency being a major driver for the development of somatic cell nuclear transfer or cloning technology, made famous through the birth of Dolly 20 years ago. Cloning technology changed how the genetic engineer planned their project. Genes could now be inserted into the gene at a given location rather than relying on the random integration associated with zygote injections (Schnieke et al. 1997). For the first time in livestock, in addition to gene addition, genes could be destroyed (knock-out animals) to generate null alleles. In addition, new transgene designs became available that could result in reduction (knockdown) of the transgene activity, for example those based on RNAi (Clark & Whitelaw, 2003).

In the first decade of experimentally modifying milk through genetic engineering strategies, genes were added to the genome. Outside of purely academic research projects, most were hoping to result in a commercial product destined for the human clinic. There was 'talk' of altering milk composition for animal nutrition and food processing goals but very little activity in this direction. With the development of new transgene designs a number of milk modifications were tested over the following two decades.

Elevated protein levels

Milk is rich in proteins. Although there are a number of proteins in milk, the majority are termed either casein or whey. Caseins are the dominant milk protein family, with most species expressing at least three different casein proteins. Cattle milk contains the calcium sensitive α- and β-casein in addition to κ-casein, the latter being common to all mammalian milk and involved in casein micelle formation and size. In addition to simply increasing the protein content in the milk, naturally elevated levels of β- and κ-casein have been linked to improved heat stability and processing properties of milk. In New Zealand, the AgResearch team led by Goetz Laible engineered cattle to have extra copies of bovine β- and κ-casein genes. These animals had 17–35% more total milk casein, with β- and κ-casein content nearly doubled compared to non-transgenic cattle (Brophy et al. 2003).

Animals over-expressing whey proteins have also been produced. In an early study at Beltsville by Bob Wall and colleagues, transgenic pigs were produced that over-expressed the mouse whey acidic protein. Although successful in expressing levels of the mouse protein in pig milk approaching those found in mouse milk, some transgenic pigs were unable to sustain lactation (Wall et al. 1991). The physiological reason for this was not determined. Subsequently, Matt Wheeler and colleagues produced transgenic pigs engineered to express a bovine α-lactalbumin transgene (Bleck et al. 1998). With initial concentrations of nearly 1 mg/ml, bovine α-lactalbumin levels were found to decrease as lactation proceeded in these pigs. These animals carried a transgene based on genomic bovine α-lactalbumin sequences and species differences in transcriptional control were proposed to account for the fold shift in bovine to porcine α-lactalbumin ratio from 4·3:1 at the start of lactation to 0·43:1 by day 20 of lactation. These animals displayed elevated lactose levels and great milk production volumes. Litters reared by transgenic gilts grew faster and were heavier than those suckling on control gilts (Noble et al. 2002; Marshall et al. 2006) and evaluation of this strategy to combat neonatal losses in the pig industry is continuing.

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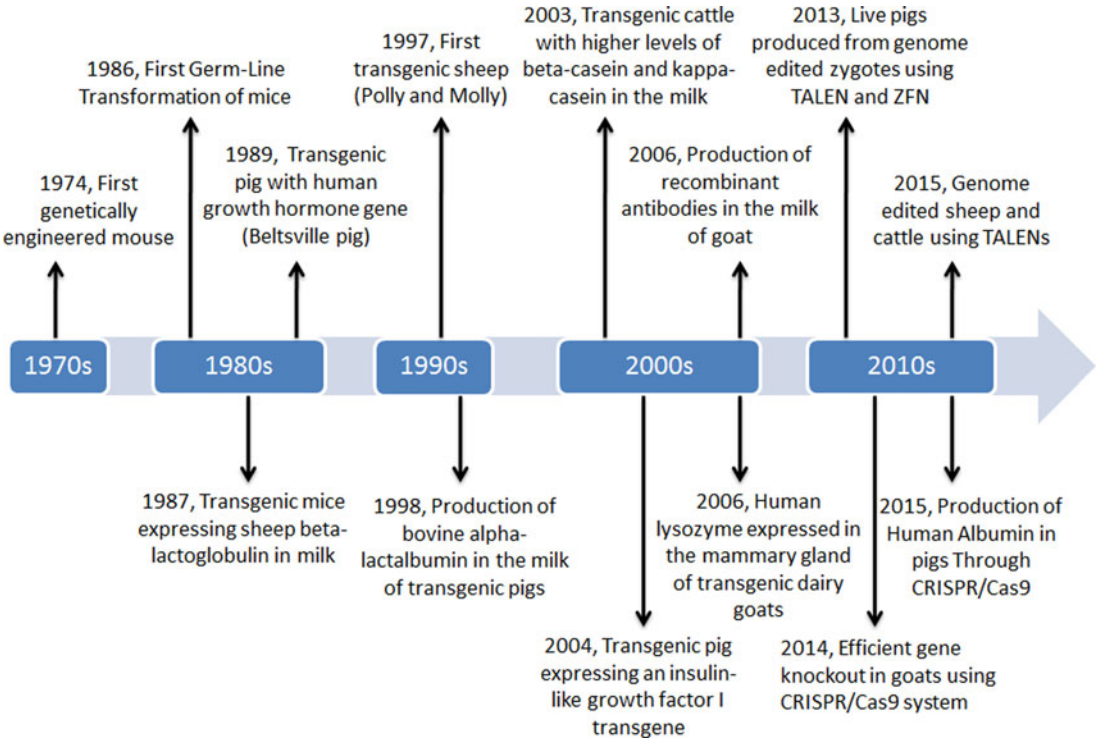

Fig. 1. A timeline of advances in genetic engineering of livestock.

In the casein over-expressing cattle, animals with the highest level of casein also displayed a slightly lower total milk protein amount (Brophy et al. 2003), reminiscent of earlier mouse work where over-expression of β-lactoglobulin in some animals corresponded with reduced casein levels reflecting what appeared to be a ceiling on total milk production, at least in some lines of transgenic mouse (McClenaghan et al. 1995). The effect of a ceiling for protein production remains to be fully understood and could have bearing on replacement and bioreactor strategies.

Another project started some years ago but now seeing rewarding progress relates to over-expression of a human lysozyme transgene in goats. Lysozyme possesses both antimicrobial properties and the ability to modulate the inflammation response. The groups of Elizabeth Maga and Jim Murray were able to produce transgenic lysozyme animals whose overall milk composition was not altered beyond the presence of human lysozyme to a level approaching that found in human milk (Maga et al. 2006). The milk did have altered properties reflected in a shorter rennet clotting time and increased curd strength which could be of benefit to the milk processing industry. Another pointer to what is possible through engineering milk. More recent data from this UC Davis team demonstrated that consumption of the lysozyme-rich milk improved intestinal and systemic piglet health (Cooper et al. 2014). Similar positive impacts on neonatal health were demonstrated for transgenic cow milk containing human lactoferrin, while earlier work indicated that cow's milk containing the bacterial enzyme lysostaphin resulted in reduced mastitis in the transgenic animals (Wall et al. 2005). These studies clearly demonstrate benefits to neonatal livestock health. In addition, Caitlin Cooper in the UC Davis team reported that consumption of the lysozyme-rich milk helped to resolve diarrhoea in piglets, suggesting that similar benefits to human health through effective treatment of Escherichia coli induced diarrhoea were likely (Cooper et al. 2013). In the future we can anticipate disease mitigation being a major target for both precision breeding and agriculture in general.

A number of other transgenic studies aiming to genetically engineer milk have been reported, ranging from reducing the levels of lactose in the milk of mice (Jost et al. 1999; Whitelaw, 1999) to altering fat composition by a stearoyl-coA desaturase transgene in goats (Reh et al. 2004). All the above studies relied on transgenic approaches that led to over-expression of milk protein or production of a novel protein in the mammary gland. Transgenic technology also enables reduction or generation of null-alleles and such approaches have seen success.

RNAi studies in the mammary gland

A powerful genetic tool relies on RNA mediated destruction of target messenger RNAs (those that encode proteins). This has proved a valuable approach to alter gene activity in plants but has proven fickle in animals – with one spectacular success. The AgResearch team led by Goetz Laible demonstrated that transgenes evoking RNA interference could have profound effects on milk composition (Jabed

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et al. 2012). They designed a microRNA that targeted both ovine and bovine β-lactoglobulin. Initially they showed that this microRNA transgene resulted in knockdown of ovine β-lactoglobulin in transgenic mice expressing this sheep protein. Subsequently, they produced cattle transgenic for the microRNA, the milk from which had barely detectable levels of β-lactoglobulin. The animals appear to have 'compensated' for this lack of a whey milk protein since they had elevated levels of the other major milk proteins. In particular, α- and β-casein were elevated two-fold, κ-casein four-fold and α-lactalbumin two-fold higher. This achievement is especially notable given the very high levels of β-lactoglobulin mRNA present in mammary epithelial cells.

What is very noticeable about all of the above projects, even those clearly demonstrating animal health benefits, is the lack of progression into the commercial sector. Many hope that it is only a matter of time for this progression to occur (Murray & Maga, 2010).

Gene knock-out

All the above studies revolved around transgene addition strategies, be it a genomic gene fragment, cDNA-based transgene or one exploiting a RNA interference strategy. Although somatic cell nuclear transfer could enable transgenic gene knock-outs (to produce a null allele), something which was achieved in biomedical orientated livestock projects, this approach was not pursued to engineer milk composition. However, such studies were successfully performed in mouse models.

The first milk protein gene to be knocked-out was achieved by Satish Kumar, who was then at the Roslin Institute (Kumar et al. 1994). Mice lacking β-casein were generated by gene targeting in mouse embryonic stem cells and produced milk with reduced micelle size. The reduction in overall casein levels due to the absence of β-casein protein was associated with a corresponding increase in the whey proteins. These changes to milk composition and physical formation resulted in reduced growth of suckled pups. No other obvious phenotypic effect on the pups was observed.

In a similar way, mice lacking α-casein have been produced by Andreas Kolb working with colleagues at the Roslin Institute (Kolb et al. 2011). Analysis of milk from these animals indicated that in addition to a lack of α-casein, levels of the remaining caseins and the whey acidic protein were all reduced, indicating that the absence of α-casein affects the secretion of all other milk proteins expressed in mammary epithelial cells. Although up-regulation of grp78, grp94 and PDIA6 proteins pointed towards involvement of endoplasmic reticulum stress, no morphological differences were observed. The reduced milk protein levels had an impact on the suckling young, resulting in reduced body weight though out life. This team subsequently demonstrated through pup cross fostering studies that non-transgenic pups suckled by α-casein deficient dams showed delayed development and reduced body weight as compare to wild type mice upon maturity (Huber et al. 2013). Thus, as with β-casein null milk, neonates suckled with α-casein null milk failed to gain full body size but otherwise developed normally.

Even more dramatic effects were produced by knocking-out κ-casein from mouse milk. This calcium insensitive phosphoglycoprotein is present in the milk of all mammals including the marsupials. Work by Satish Kumar, now at CCMB in Hyderabad, won the race to produce κ-casein gene knock-out mice (Shekar et al. 2006). These animals did not produce any κ-casein in their mammary gland, with expression of the other casein genes remaining unaffected, and κ-casein null females were viable, fertile and carried pregnancy to term. However, females were unable to let down milk even after oxytocin injections. Although milk was produced within the mammary gland, it coagulated in situ and was retained (blocking) in the gland lumina. A similar outcome, the inability to sustain offspring through suckling, was observed for α-lactalbumin knock-out mice (Stacey et al. 1995).

The new genome editing tools

We are entering a new era for modifying milk by genetic engineering approaches. An era where there is the real likelihood that multiple genetically engineered livestock projects will progress from the laboratory to the farm and thus into our food chain. The goals haven't changed; to produce more milk whilst simultaneously optimising animal health, to produce more appropriate milk products, and to establish alternative dairy usage. This renewed enthusiasm is based on new technology, with the advent of the genome editors now enabling efficient and precise changes to the genomes of livestock (Fahrenkrug et al. 2010). For example, DNA deletions or insertions, or DNA base exchanges can be achieved, and in this way alleles can be made or removed from a given population (Tan et al. 2013; Fig. 2).

What are genome editors?

At their simplest, genome editors are precision tools that can be used to target a specific location in the DNA of a cell, creating a break in the DNA at that position. To put this into context, the bovine genome is approximately 3 billion bases long; targeting a specific location in this genome is akin to locating a single letter from a stack of over 800 King James bibles. There are currently three main types of genome editor, and each works in a slightly different way. Zinc fingers are naturally occurring small protein motifs, many of which have DNA binding functions. The Cys_2His_2 zinc fingers currently used bind three specific DNA bases, and can be organised into arrays that recognise larger stretches of DNA. These zinc finger arrays are in turn fused to the nuclease domain of the obligate dimer FokI, a

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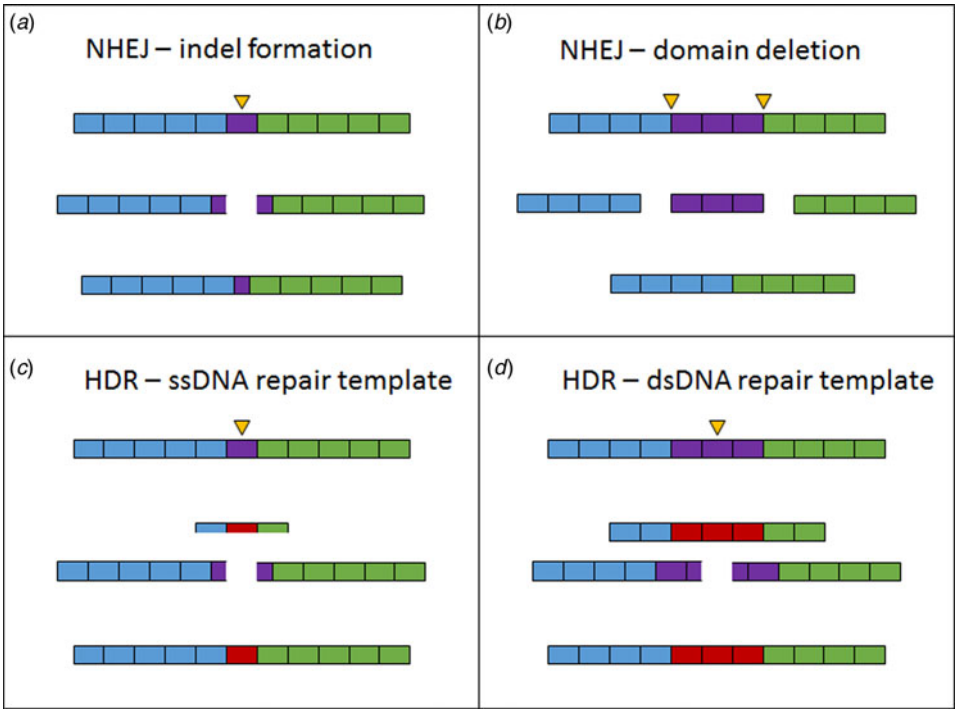

Fig. 2. Schematic indicating how genome editing works. In each section: target genomic DNA (upper) with the genome editor cutting site depicted (arrowhead), genome editor digested double strand break without or with template DNA (middle), and edited genomic sequence (lower). Genome editors inducing NHEJ can cause small insertions or deletions (A) or larger deletions (B), and by inducing HDR enable sequence conversion using single stranded DNA oligo (C) or double stranded DNA plasmid (D). NHEJ: non-homologous end joining. HDR: homology directed repair.

restriction endonuclease, forming a zinc-finger nuclease (ZFN). ZFNs are employed as pairs that between them recognise between 18 and 32 bases of DNA, with each member of the pair binding one or other strand of the DNA double helix. Binding of a pair of ZFNs to their designated target site results in the 2 halves of the FokI dimerising on the intervening sequence and cutting the DNA. ZFNs have been used to aid in modification of the bovine genome (Liu et al. 2014b), but in general their uptake by the livestock research community has been relatively low, limited by complexities in their design.

Transcription activator-like effectors (TALEs) are proteins used by proteobacteria of the genus Xanthomonas to subvert the transcriptional activity in the cells of their plant hosts. These proteins are composed of an array of DNA-binding modules, with each module having specificity for binding a single DNA base. Scientists have utilised this 1-to-1 relationship between protein modules and DNA bases to build their own protein arrays capable of binding almost any sequence within the genome. As with ZFNs, the TALE array is fused to the FokI nuclease to give a TALEN, and as with ZFNs these are employed in pairs to allow dimerisation of FokI to give targeted cutting. TALENS have been more widely utilised in livestock research than ZFNs, largely due to their relative ease of design and synthesis (Tan et al. 2013; Proudfoot et al. 2015; Wu et al. 2015).

The CRISPR/Cas system utilised by scientists has been derived from a bacterial innate immune system that allows the bacteria to rapidly respond to previously-encountered pathogens such as bacteriophage or plasmids. This genome editing tool has significant mechanistic differences from both TALENs and ZFNs; while both of the aforementioned tools use protein DNA-binding motifs to guide a fused nuclease to the target site, the CRISPR/Cas system utilises Watson-Crick base pairing between a short guide RNA (20 nucleotides in length) and its cognate target sequence to direct a complexed nuclease (currently most typically the Cas9 nuclease) to cut at the said site. CRISPR/Cas is arguably the simplest of the genome editor systems to use, and as such has seen a very rapid uptake by the research community since the seminal publications by both the Zhang and Jaenisch labs in early 2013 (Cong et al. 2013; Yang et al. 2013).

Cellular repair of the breaks introduced by genome editors is predominantly by the non-homologous end joining (NHEJ) pathway, widely considered to be error prone, the consequence of which is the introduction of small insertions or deletions (indels) at the cut site; if that site is within the coding sequence of a gene then the outcomes range from insertion, deletion or modification of single amino acids (the building blocks of proteins) to frameshift events and functional gene knockout. Refinement of editor application to include a DNA template molecule

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can result in the cell performing homology-directed repair rather than NHEJ, meaning that specific rather than random changes can be introduced. These can take the form of anything from single base changes to allele exchange. Alternatively, editors can be applied in pairs to effectively delete the chosen sequence between them from the genome. This allows researchers to remove either entire genes or specific portions of a gene.

With regard to milk production, this new genetic technology, based on the genome editors which enable precision breeding, can be applied to all dairy species. It has been achieved in cattle (Proudfoot et al. 2015), sheep (Proudfoot et al. 2015; Crispo et al. 2015) and goats (Ni et al. 2014); it can only be a matter of time before genome edited buffalo are produced.

What could be done – the opportunities

There are a variety of ways genome editing technology can be used in regard to engineering milk. First, if desired, all the studies in mice utilising transgenic gene knockout strategies could be repeated using the genome editors. Except in this way the animals would not be transgenic but merely carry a specific mutation at a precise site within the target gene which results in gene inactivation. This could be achieved through the production of a NHEJ enabled frame-shift mutation causing an otherwise out-of-frame stop codon to be translated. This can be efficiently achieved in livestock as demonstrated by Simon Lillico and colleagues for the porcine RELA gene (Lillico et al. 2013). An alternative strategy could be to delete the transcriptional start site and/or the translational AUG codon, thus inactivating the target gene.

The gene knock-out mouse studies indicate that the calcium sensitive caseins could be removed, at least individually, and lactation maintained. However, in these animals pup growth was impaired, although the smaller resultant animals were otherwise normal in development and behaviour. It is possible that reduced milk protein levels achieved through a NHEJ indel mutation could be complemented by the coincident insertion of a transgene, thus engineering the production of other proteins (including other milk proteins) to bring total proteins levels back to normal levels. Dependent on the actual protein, this should be able to maintain both normal lactation and offspring growth.

Potentially more powerful, HDR can be evoked to engineer more subtle and predetermined changes to the genome. Through supply of the appropriate template DNA with the genome editor, HDR can be used to produce different alleles, even entire haplotypes. This has been achieved in pigs for a candidate gene involved in host resilience to a virus (Lillico et al, in press). Goetz Laible, Scott Fahrenkrug and colleagues have indicated how this could be used to engineer milk. Building on their success with an RNAi transgene to knock-down β-lactoglobulin activity, this group used both ZFN and TALEN reagents to inactivate the bovine β-lactoglobulin gene through an HDR event (Wei et al. 2015). Although this project has still to progress to testing in animals, it clearly points the way to inactivating any of the milk protein genes in livestock. Given that β-lactoglobulin is considered an allergen, producing cattle milk which lacked this protein could considerably increase the use of this animal product as a substitute for human milk. It is estimated that 2–3% of infants are allergic to cow's milk during the first year of their life. This study indicates that it should be easy to engineer milk by exchange of alleles associated with different milk properties or alleles associated with great milk production potential. There is also the possibility of engineering milk protein genes to optimise availability of bioactive peptides released during digestion in the gut (Nongonierma & FitzGerald, 2015). This could be through capturing genetic variation in the milk protein genes (Caroli et al. 2009), including that variation underlying the unique antimicrobial properties of monotremes (Enjapoori et al. 2014). An intriguing extension to this line of thought would be engineering vaccines into milk, the feasibility of which has been demonstrated through the production of antibodies in transgenic milk (Sola et al. 1998; Kolb et al. 2001). Further, allelic variation which confers altered physical properties and thus facilitates the processing of milk into various dairy products can be envisaged. We can glean from the numerous transgenic and milk protein knockout studies performed in mice, that considerable changes in milk composition can be tolerated without interfering with micelle formation. Therefore, there is considerable scope of the rational engineering of milk for downstream processing.

Most studies to-date with regard to engineering milk have focussed on known genes. With the continuing reduction of genome sequencing cost (Desai et al. 2012), we can anticipate knowledge of the genetic variation impacting on milk to dramatically expand. To illustrate the potential, identification of the genetic variation controlling lactation may come from comparison of the underlying genetics which controls the extreme phenotypic differences in milk production between Bos taurus and Bos indicus cattle. Precision breeding through the use of genome editors would enable introgression of favourable alleles (as is being attempted for virus resilience in pigs: (Lillico et al. 2013). This could have major rewards with regard to Bos indicus milk production in the tropical regions of the world.

There are different ways to utilise genome editors. The production of small indels or mutations is elegant in comparison to the insertion of a transgene; however there may be cases where the latter is preferable. It has become apparent that the individual animals often carry varying numbers of copies of a gene. This is termed copy number variation and may contribute to disease in some cases (Clop et al. 2012). Analogously, the casein locus displays an ancestral copy number variation through the duplication of the calcium-sensitive casein genes (Rijnkels et al. 2003) but in this case the extra copies are presumably beneficial, resulting in increased milk protein levels. Genome editing technology could be deployed to continue this normal

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evolutionary process by enabling the targeted integration of extra milk protein gene copies into the genome. Targeting could be to a permissive locus, equivalent to the mouse Rosa locus, or adjacent to a milk protein gene. To continue this theme, genome editors are already being used to progress the animal bioreactor concept by enabling efficient and precise transgene integration (Liu et al. 2014a; Cui et al. 2015; Peng et al. 2015).

The future for engineered milk

Food security for all is an ambitious challenge, yet one that is essential for our society to stably migrate through the coming century. In 2000, the FAO predicted that the world will consume 700 m tonnes of dairy protein products annually by 2020. This level of dairy production has already been reached. Can we expect this production increase to continue: yes, if the industry continues to innovate and agriculture becomes more efficient. Genetic engineering technology can contribute to this goal.

The next few years will be exciting in this field, for there are multiple opportunities (Fig. 3). For human benefit, perhaps cattle producing milk without the major allergen β-lactoglobulin and a raft of biomedical proteins, helping to treat patients, produced in livestock milk. To benefit animals on the farm, milk with higher levels of protein could be used to enhance neonatal survival and welfare. This would obviously benefit the dairy industry and consumer alike. Alternatively we can consider replacing the endogenous milk protein genes of livestock with their human counterpart to 'humanise' the milk for human consumption. As scientists we have all the tools we need to achieve this experimentally. The challenge of translating these successes to the farm and into the food chain remains.

The first genetically engineered animal to navigate its way through the regulatory pathway and closest to entering our food chain is the Aquadvantage salmon™ (recently gaining FDA approval on the 19th November 2015). In parallel, the European Food Safety Authority have published guidelines for taking a GM livestock product through to market (http://www.efsa.europa.eu/en/efsajournal/pub/32iu00). Others also contribute to establishing the environment for this to happen, for example BIO's good stewardship guidelines (https://www.bio.org/articles/bio-guidance-genetically-engineered-animal-stewardship). In the UK, the government's Agritech strategy embraces new technologies including genetic engineering (https://www.gov.uk/government/collections/agricultural-technologies-agri-tech-strategy).
Similar opportunities to promote agriculture are appearing around the world in response to the global challenge of food security. The platform for genetically engineered livestock with enhanced milk is in place. This is not to imply that this technology is the only way forward. With regards to livestock, genetic selection, better husbandry, optimal feed supply, effective vaccines and available drugs, and many other aspects will positively impact on agriculture.

Fig. 3. Options for engineering milk.

We can also anticipate significant advance in dairy processing, product type and distribution. We will need many solutions to provide enough, nutritious and safe food for our societies.

There are counter activities, for example the diverse debate on labelling in relation to GM foods in the US. For those of us in the European Union, member states can unilaterally choose to ban GM foodstuffs. Although this is not based on scientific evidence, it could currently be in the interest of an individual member state based on political stances to ban GM food production, in the longer term such strategies run the real risk of 'second citizen' status for agriculture in those countries. Equally important is whether the citizen will buy such products if approved and on the shop shelf. Here dialogue is needed to allow debate on concerns, risk and benefits to be regularly discussed. For this to be successful all stakeholders need to be involved and each must come with an open mind, prepared to change opinion based upon scientific evidence and accept compromise as the dialogue progresses. For precision breeding using genome editing technology, this dialogue will complement that which is starting to consider the use of these tools in humans (Lanphier et al. 2015; Mathews et al. 2015) and that of the non-regulated random mutagenic strategies which have been widely used for some time in plant agriculture (Schaeffer & Nakata, 2015).

Finally, we return to our comment above, we believe "we have all the tools". It is up to those of us who work in the field of milk production to imagine and experimentally devise, then transparently and reproducibly evaluate what are useful milk-oriented genetic engineering projects. We need to robustly demonstrate the utility and benefit to

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both animal production and welfare, and to the consumer, and identify any potential risks. Then society can choose to use or not.

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References

Anon 2009 Recombinant human Antithrombin (ATryn). *Medical Letter on Drugs and Therapeutics* **51** 83–84

Bleck GT, White BR, Miller DJ & Wheeler MB 1998 Production of bovine alpha-lactalbumin in the milk of transgenic pigs. *Journal of Animal Science* **76** 3072–3078

Brophy B, Smolenski G, Wheeler T, Wells D, L'Huillier P & Laible G 2003 Cloned transgenic cattle produce milk with higher levels of beta-casein and kappa-casein. *Nature Biotechnology* **21** 157–162

Caroli AM, Chessa S & Erhardt GJ 2009 Invited review: milk protein polymorphisms in cattle: effect on animal breeding and human nutrition. *Journal of Dairy Science* **92** 5335–5352

Clark J & Whitelaw B 2003 A future for transgenic livestock. *Nature Reviews Genetics* **4** 825–833

Clark AJ, Ali S, Archibald AL, Bessos H, Brown P, Harris S, McClenaghan M, Prowse C, Simons JP, Whitelaw CB & Wilmut I 1989 The molecular manipulation of milk composition. *Genome* **31** 950–955

Clop A, Vidal O & Amills M 2012 Copy number variation in the genomes of domestic animals. *Animal Genetics* **43** 503–517

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA & Zhang F 2013 Multiplex genome engineering using CRISPR/Cas systems. *Science* **339** 819–823

Cooper CA, Nelson KM, Maga EA & Murray JD 2013 Consumption of transgenic cows' milk containing human lactoferrin results in beneficial changes in the gastrointestinal tract and systemic health of young pigs. *Transgenic Research* **22** 571–578

Cooper CA, Maga EA & Murray JD 2014 Consumption of transgenic milk containing the antimicrobials lactoferrin and lysozyme separately and in conjunction by 6-week-old pigs improves intestinal and systemic health. *Journal of Dairy Research* **81** 30–37

Cooper CA, Maga EA & Murray JD 2015 Production of human lactoferrin and lysozyme in the milk of transgenic dairy animals: past, present, and future. *Transgenic Research* **24** 605–614

Crispo M, Mulet AP, Tesson L, Barrera N, Cuadro F, dos Santos-Neto PC, Nguyen TH, Creneguy A, Brusselle L, Anegon I & Menchaca A 2015 Efficient generation of Myostatin knock-out sheep using CRISPR/Cas9 technology and microinjection into zygotes. *PLoS One* **10** e0136690

Cui C, Song Y, Liu J, Ge H, Li Q, Huang H, Hu L, Zhu H, Jin Y & Zhang Y 2015 Gene targeting by TALEN-induced homologous recombination in goats directs production of beta-lactoglobulin-free, high-human lactoferrin milk. *Scientific Reports* **5** 10482

Desai N, Antonopoulos D, Gilbert JA, Glass EM & Meyer F 2012 From genomics to metagenomics. *Current Opinions in Biotechnology* **23** 72–76

Enjapoori AK, Grant TR, Nicol SC, Lefevre CM, Nicholas KR & Sharp JA 2014 Monotreme lactation protein is highly expressed in monotreme milk and provides antimicrobial protection. *Genome Biology and Evolution* **6** 2754–2773

Fahrenkrug SC, Blake A, Carlson DF, Doran T, Van Eenennaam A, Faber D, Galli C, Gao Q, Hackett PB, Li N, Maga EA, Muir WM, Murray JD, Shi D, Stotish R, Sullivan E, Taylor JF, Walton M, Wheeler M, Whitelaw B & Glenn BP 2010 Precision genetics for complex objectives in animal agriculture. *Journal of Animal Science* **88** 2530–2539

FAO 2013 http://reliefweb.int/sites/reliefweb.int/files/resources/FAO_2013_stats_yrbook.pdf

Hammer RE, Pursel VG, Rexroad CE, Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD & Brinster RL 1985 Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* **315** 680–683

Houdebine LM 2000 Transgenic animal bioreactors. *Transgenic Research* **9** 305–320

Huber RC, Kolb AF, Lillico S, Carlisle A, Sandoe P, Sorensen DB, Remuge L, Whitelaw BC & Olsson AI 2013 Behaviour of postnatally growth-impaired mice during malnutrition and after partial weight recovery. *Nutritional Neuroscience* **16** 125–134

Jabed A, Wagner S, McCracken J, Wells DN & Laible G 2012 Targeted microRNA expression in dairy cattle directs production of beta-lactoglobulin-free, high-casein milk. *Proceedings of the National Academy of Sciences of the USA* **109** 16811–16816

Jost B, Vilotte JL, Duluc I, Rodeau JL & Freund JN 1999 Production of low-lactose milk by ectopic expression of intestinal lactase in the mouse mammary gland. *Nature Biotechnology* **17** 160–164

Kind A & Schnieke A 2008 Animal pharming, two decades on. *Transgenic Research* **17** 1025–1033

Kolb AF, Webster J, Whitelaw CB & Siddell SG 2001 A virus-neutralising monoclonal antibody expressed in the milk of transgenic mice. *Advances in Experimental Medicine and Biology* **494** 411–414

Kolb AF, Huber RC, Lillico SG, Carlisle A, Robinson CJ, Neil C, Petrie L, Sorensen DB, Olsson IA & Whitelaw CB 2011 Milk lacking alpha-casein leads to permanent reduction in body size in mice. *PLoS ONE* **6** e21775

Kumar S, Clarke AR, Hooper ML, Horne DS, Law AJ, Leaver J, Springbett A, Stevenson E & Simons JP 1994 Milk composition and lactation of beta-casein-deficient mice. *Proceedings of the National Academy of Sciences of the USA* **91** 6138–6142

Lanphier E, Urnov F, Haecker SE, Werner M & Smolenski J 2015 Don't edit the human germ line. *Nature* **519** 410–411

Lillico SG, Proudfoot C, Carlson DF, Stverakova D, Neil C, Blain C, King TJ, Ritchie WA, Tan W, Mileham AJ, McLaren DG, Fahrenkrug SC & Whitelaw CB 2013 Live pigs produced from genome edited zygotes. *Scientific Reports* **3** 2847

Liu X, Ping H & Zhang C 2014a Rapid establishment of a HEK 293 cell line expressing FVIII-BDD using AAV site-specific integration plasmids. *BMC Research Notes* **7** 626

Liu X, Wang Y, Tian Y, Yu Y, Gao M, Hu G, Su F, Pan S, Luo Y, Guo Z, Quan F & Zhang Y 2014b Generation of mastitis resistance in cows by targeting human lysozyme gene to beta-casein locus using zinc-finger nucleases. *Proceedings of the Royal Society B: Biological Sciences* **281** 20133368

Lubon H, Paleyanda RK, Velander WH & Drohan WN 1996 Blood proteins from transgenic animal bioreactors. *Transfusion Medicine Reviews* **10** 131–143

Maga EA, Cullor JS, Smith W, Anderson GB & Murray JD 2006 Human lysozyme expressed in the mammary gland of transgenic dairy goats can inhibit the growth of bacteria that cause mastitis and the cold-spoilage of milk. *Foodborne Pathogens and Disease* **3** 384–392

Marshall KM, Hurley WL, Shanks RD & Wheeler MB 2006 Effects of suckling intensity on milk yield and piglet growth from lactation-enhanced gilts. *Journal of Animal Science* **84** 2346–2351

Mathews DJ, Chan S, Donovan PJ, Douglas T, Gyngell C, Harris J, Regenberg A & Lovell-Badge R 2015 CRISPR: a path through the thicket. *Nature* **527** 159–161

McClenaghan M, Springbett A, Wallace RM, Wilde CJ & Clark AJ 1995 Secretory proteins compete for production in the mammary gland of transgenic mice. *Biochemal Journal* **310** 637–641

Murray JD & Maga EA 2010 Is there a risk from not using GE animals? *Transgenic Research* **19** 357–361

Ni W, Qiao J, Hu S, Zhao X, Regouski M, Yang M, Polejaeva IA & Chen C 2014 Efficient gene knockout in goats using CRISPR/Cas9 system. *PLoS One* **9** e106718

Noble MS, Rodriguez-Zas S, Cook JB, Bleck GT, Hurley WL & Wheeler MB 2002 Lactational performance of first-parity transgenic gilts expressing bovine alpha-lactalbumin in their milk. *Journal of Animal Science* **80** 1090–1096

Nongonierma AB & FitzGerald RJ 2015 Bioactive properties of milk proteins in humans: a review. *Peptides* **73** 20–34

Palmiter RD & Brinster RL 1986 Germ-line transformation of mice. *Annual Review of Genetics* **20** 465–499

Peng J, Wang Y, Jiang J, Zhou X, Song L, Wang L, Ding C, Qin J, Liu L, Wang W, Liu J, Huang X, Wei H & Zhang P 2015 Production of human albumin in pigs through CRISPR/Cas9-mediated Knockin of human cDNA into swine albumin locus in the Zygotes. *Scientific Reports* **5** 16705

Pollock DP, Kutzko JP, Birck-Wilson E, Williams JL, Echelard Y & Meade HM 1999 Transgenic milk as a method for the production of recombinant antibodies. *Journal of Immunological Methods* **231** 147–157

Proudfoot C, Carlson DF, Huddart R, Long CR, Pryor JH, King TJ, Lillico SG, Mileham AJ, McLaren DG, Whitelaw CB & Fahrenkrug SC 2015 Genome edited sheep and cattle. *Transgenic Research* **24** 147–153

Pursel VG, Pinkert CA, Miller KF, Bolt DJ, Campbell RG, Palmiter RD, Brinster RL & Hammer RE 1989 Genetic engineering of livestock. *Science* **244** 1281–1288

Pursel VG, Bolt DJ, Miller KF, Pinkert CA, Hammer RE, Palmiter RD & Brinster RL 1990 Expression and performance in transgenic pigs. *Journal of Reproduction and Fertility Supplement* **40** 235–45

Pursel VG, Mitchell AD, Bee G, Elsasser TH, McMurtry JP, Wall RJ, Coleman ME & Schwartz RJ 2004 Growth and tissue accretion rates of swine expressing an insulin-like growth factor I transgene. *Animal Biotechnology* **15** 33–45

Qian L, Tang M, Yang J, Wang Q, Cai C, Jiang S, Li H, Jiang K, Gao P, Ma D, Chen Y, An X, Li K & Cui W 2015 Targeted mutations in myostatin by zinc-finger nucleases result in double-muscled phenotype in Meishan pigs. *Scientific Reports* **5** 14435

Reh WA, Maga EA, Collette NM, Moyer A, Conrad-Brink JS, Taylor SJ, DePeters EJ, Oppenheim S, Rowe JD, BonDurant RH, Anderson GB & Murray JD 2004 Hot topic: using a stearoyl-CoA desaturase transgene to alter milk fatty acid composition. *Journal of Dairy Science* **87** 3510–3514

Rijnkels M, Elnitski L, Miller W & Rosen JM 2003 Multispecies comparative analysis of a mammalian-specific genomic domain encoding secretory proteins. *Genomics* **82** 417–432

Schaeffer SM & Nakata PA 2015 CRISPR/Cas9-mediated genome editing and gene replacement in plants: transitioning from lab to field. *Plant Science* **240** 130–142

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A & Campbell KH 1997 Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* **278** 2130–2133

Shekar PC, Goel S, Rani SD, Sarathi DP, Alex JL, Singh S & Kumar S 2006 kappa-casein-deficient mice fail to lactate. *Proceedings of the National Academy of Sciences of the USA* **103** 8000–8005

Simons JP, McClenaghan M & Clark AJ 1987 Alteration of the quality of milk by expression of sheep beta-lactoglobulin in transgenic mice. *Nature* **328** 530–532

Sola I, Castilla J, Pintado B, Sanchez-Morgado JM, Whitelaw CB, Clark AJ & Enjuanes L 1998 Transgenic mice secreting coronavirus neutralizing antibodies into the milk. *Journal of Virology* **72** 3762–3772

Stacey A, Schnieke A, Kerr M, Scott A, McKee C, Cottingham I, Binas B, Wilde C & Colman A 1995 Lactation is disrupted by alpha-lactalbumin deficiency and can be restored by human alpha-lactalbumin gene replacement in mice. *Proceedings of the National Academy of Sciences of the USA* **92** 2835–2839

Tan W, Carlson DF, Lancto CA, Garbe JR, Webster DA, Hackett PB & Fahrenkrug SC 2013 Efficient nonmeiotic allele introgression in livestock using custom endonucleases. *Proceedings of the National Academy of Sciences of the USA* **110** 16526–16531

Wall RJ, Pursel VG, Shamay A, McKnight RA, Pittius CW & Hennighausen L 1991 High-level synthesis of a heterologous milk protein in the mammary glands of transgenic swine. *Proceedings of the National Academy of Sciences of the USA* **88** 1696–1700

Wall RJ, Powell AM, Paape MJ, Kerr DE, Bannerman DD, Pursel VG, Wells KD, Talbot N & Hawk HW 2005 Genetically enhanced cows resist intramammary Staphylococcus aureus infection. *Nature Biotechnology* **23** 445–451

Wei J, Wagner S, Lu D, Maclean P, Carlson DF, Fahrenkrug SC & Laible G 2015 Efficient introgression of allelic variants by embryo-mediated editing of the bovine genome. *Scientific Reports* **5** 11735

Whitelaw B 1999 Toward designer milk. *Nature Biotechnology* **17** 135–136

Wilmut I & Whitelaw CB 1994 Strategies for production of pharmaceutical proteins in milk. *Reproduction, Fertility and Development* **6** 625–630

Wu H, Wang Y, Zhang Y, Yang M, Lv J, Liu J & Zhang Y 2015 TALE nickase-mediated SP110 knockin endows cattle with increased resistance to tuberculosis. *Proceedings of the National Academy of Sciences of the USA* **112** E1530–E1539

Yang H, Wang H, Shivalila CS, Cheng AW, Shi L & Jaenisch R 2013 One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* **154** 1370–1379

Zhao J, Xu W, Ross JW, Walters EM, Butler SP, Whyte JJ, Kelso L, Fatemi M, Vanderslice NC, Giroux K, Spate LD, Samuel MS, Murphy CN, Wells KD, Masiello NC, Prather RS & Velander WH 2015 Engineering protein processing of the mammary gland to produce abundant hemophilia B therapy in milk. *Scientific Reports* **5** 14176

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