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Review

Transgenesis in large domestic species: future development for milk modification

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Abstract — The present review has two goals. First, to offer an overview of recent advances in the technical strategies applied to the production of transgenic large domestic animals, and second, to review how transgenic technology can be applied to the modification of milk composition. Transgenic sheep and cattle obtained through nuclear transfer are now a reality, opening up a means of ruminant transgenic production with an efficiency that entitles us to consider it a serious alternative to microinjection. Nuclear transfer also consistently reduces the time needed to establish a transgenic production herd, and what is more important, it opens up the way to homologous recombination in large species, which at the moment is restricted to mice. Other interesting technological contributions have also taken place lately, some of them towards the modification of the male germ line, and others developing viral vectors with the ability to alter the genetic information of animals. The simplification of the methodology and the consistent reduction of the time needed to carry out a transgenic experiment will allow us to test several hypotheses directed at the modification of milk components. This may help towards the application of transgenic technology in the dairy industry, which unlike pharmaceutical companies, has been somehow reluctant over these approaches.

transgenesis / milk / dairy industry / cattle

Résumé — Transgenèse chez les grands mammifères domestiques, développement à venir de la modification du lait. Cet article a deux objectifs. Premièrement, de proposer une revue des avancées récentes en matière de stratégies appliquées à la production d'animaux transgéniques chez les grands mammifères domestiques et, deuxièmement, d'examiner jusqu'à quel point la transgenèse peut être appliquée aux modifications de la composition du lait. L'obtention d'ovins et de bovins transgéniques est maintenant une réalité ouvrant une porte à la production d'animaux transgéniques avec une efficacité telle, qu'elle permet de considérer cette technique comme une alternative sérieuse à la micro-injection. De plus, le transfert nucléaire réduit considérablement l'intervalle nécessaire à l'établissement d'un troupeau d'animaux transgéniques et, ce qui est plus important, permet d'envisager la recombinaison homologue, jusque là restreinte à la souris, chez les grands mammifères.

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D'autres technologies intéressantes se sont développées, telles que la modification de la lignée germinale et le développement de vecteurs viraux capables d'altérer l'information génétique des animaux. La simplification de la méthodologie et la réduction constante du temps nécessaire aux expériences de transgenèse permettront de tester diverses hypothèses concernant la modification des composants du lait. Ceci facilitera l'application des technologies de la transgenèse dans l'industrie laitière qui, contrairement à l'industrie pharmaceutique, a été quelque peu réticente à ces approches. © Inra/Elsevier, Paris

transgenèse / lait / industrie laitière / bovins

1. INTRODUCTION

Global milk production in cattle has increased since the early 1940s as a consequence of genetic selection; however, over the last few years, it has reached a plateau. Several causes can be indicated: a decrease in the number of milking animals, a smaller profit derived from an unfavourable milk price/production cost ratio, and, specifically in the European Union, as a consequence of the quota restraints imposed [13, 14]. This encourages us to consider an increase in the milk quality a unique way both to obtain new markets and reduce costs in the manufacture of dairy products.

As a result of genetic selection over the past decades, the dairy industry has achieved a remarkable improvement in milk production. Nevertheless, the genetic gain which can now be expected based on traditional selection schemes is slow owing to the intergeneration time. It is also limited by the low hereditability of these traits, the interrelations among them, and because different milk products may benefit from selection in different directions [16]. The increased production, however, has not been accompanied by significant changes in milk composition. Modification of quality would be dictated by the market demand, because only a financial incentive may justify a change. Nevertheless, the increased knowledge of the bovine genome, and specifically of the milk protein genes [19, 41] has provided new tools that allow a more efficient selection to increase quality traits of milk composition. This can be focused on a general characteristic, such as increasing the total amount of protein in milk [45], or it can be directed towards a specific trait, such as selecting cattle carrying the κ-casein B allele.

Transgenesis can be defined as the modification of the genetic information of an organism through techniques of DNA recombination, and it represents a new step towards altering milk composition. It has opened a full range of possibilities unavailable to the classical selection schemes, which are limited to the set of genes present in the bovine genome. Even though combinations of these genes are almost endless, some characteristics are out of reach. We intend to review here how transgenesis can contribute to the modification of milk, and also to summarize the progress and future perspectives of the methods that have made this manipulation possible but that somehow have limited its development.

2. TECHNICAL ASPECTS OF TRANSGENESIS: RECENT EVOLUTION AND FUTURE PERSPECTIVES

If something has changed in this field during the last 3 years it is the perspective of how a transgenic project in cattle can be approached. Dolly, the first animal obtained from the nuclear transplant of an adult cell [62], has gained worldwide recognition. However, in this particular field, Polly, the first transgenic sheep obtained through nuclear transfer [51], and George and

Charlie [9], calves obtained in the same way, are the real milestones and will lead the way in the future. Cloning has turned into a real complement to microinjection because it consistently allows a shortening of the time between the obtention of a founder and the establishment of a lactation herd. But this is not the only advantage, nuclear transfer opens a way to homologous recombination and knock-in/knock-out technologies, which, till now, have been restricted to mice.

Pronuclear microinjection, the only reliable method of producing large transgenic animals up to 1997, was first applied to mice embryos [18] and later adapted to livestock embryos [23]. Since then it has been restricted to a very small number of laboratories with enough technical and economical potential to accomplish this research. Microinjection of in vitro produced bovine embryos [31] substituted a more expensive approach, consisting in the recovery of embryos at the pronuclear stage, either surgically or after slaughter of superovulated donor cows. This in vitro source of embryos made microinjection accessible to those laboratories that lacked the facilities to keep donor cows. However, there was no solution to the necessity of producing more than 100 pregnancies from injected embryos in order to obtain a founder animal [59], that is, an animal that has incorporated the transgene into its genome and is able to pass it to the next generation.

Efficiency of microinjection in large domestic animals is very low [60, 61], and the costs derived from keeping so many animals have dissuaded many researchers from testing their hypothesis in the species of choice. This justifies why most of the approaches towards altering milk composition have been mostly carried out in mice, and to a lesser extent in rabbits or pigs.

Nevertheless, some steps have been taken towards increasing the efficiency of microinjection, such as reducing the number of recipients needed by the identification of the 'transgenic embryos' before transfer. Detection of the presence of the injected DNA in early stage embryos by PCR has been proposed [44]. However, this technique was unable to differentiate the true transgenic embryos owing to the long permanence of microinjected DNA in unintegrated form, which can be several days. Other selection approaches have focused on trying to detect the activity of reporter genes that would only be expressed if the whole construct had been integrated into the genome. Some have been based on the expression of an antibiotic resistance [2, 57], the detection of luciferase [39, 42] or green fluorescence protein (GFP) [58]. The latter protein is easy to detect but unfortunately its expression is not restricted to stable integrated DNA, and hence both false positives and false negatives can be found. In an attempt to develop a true reporter of integration that would allow us to differentiate between the expression resulting from a stable integration and that from a transient one, we added MAR sequences to a reporter gene based on expression of GFP under the control of different promoters. However, the long half life of this protein in embryonic cells only allowed an increase in the number of transgenic progeny after the transfer of green embryos when selection was performed at the two-cell stage [47].

Nuclear transfer has indirectly solved this situation. This technique makes possible the transfer of only true transgenic embryos because it is performed using the nucleus of a cell selected in base to the transgene integration. For this reason any individual obtained from these reconstructed embryos will be transgenic and no further selection of embryos is needed.

As we mentioned above, nuclear transfer provides another advantage of higher transcendence, the possibility of direct integration into a specific genome region. With microinjection, transgenes are randomly integrated into any part of the genome. This means that they can disrupt essential genes or be located in chromosomal regions inac-

cessible to transcription and translation, and therefore, they would never be expressed. On the other hand, microinjected constructs can only provide extra information, that is, they only add information to what is already present in the genome. When the goal is to suppress the activity of a given endogenous gene, it has to be accomplished with indirect strategies such as antisense RNA or ribozymes. Nuclear transfer can benefit from all the advantages of knock-out strategies which have allowed us to target the activity of endogenous genes in mice. To date, knock-out cattle have not yet been produced, but it is just a question of time before we have a transformable pluripotent cell line available in cattle.

Recently, transgenic cattle have been produced for the first time by means of a viral vector [8] targeted towards the oocyte. Even though the system is restricted to transgenes of limited size, owing to the limitations of a viral vector, it provides an alternative method, at least for those species in which in vitro fertilization is possible.

Another technique reviewed recently [15] and still controversial is the use of spermatozoa as vectors of exogenous DNA [33]. A recent experiment in this field [36] shows that even today a great disparity among results can be obtained with the same protocol within different laboratories and even within the same laboratory. Spermatozoa are not the only male germinal cells susceptible to be used in the production of transgenics. In 1994 Dr Brinster led a series of works that demonstrated how spermatogonia, a cell type precursor of spermatozoa, can be collected from one male and transferred into the testicle of another male of the same [5, 6] or different species [10], and become functional. This opens the possibility of including exogenous genetic information in these cells prior to transfer. In situ transformation of male germ lines has also been reported [30, 50, 63] through direct DNA injection into the testicle of living animals. This may prove to be an alternative of interest for those species in which the transplant of spermatogonia into the seminiferous tubules is technically impossible.

After this overview of different approaches to producing transgenic animals, we can expect an increased efficiency in the methodology and, as a result, more experiments designed for large animals providing quick solutions to the consumer's demands and this, perhaps, will help to change their negative perception of transgenic products.

3. THE INDUSTRIAL INPUT

Most of the more important contributions directed towards the modification of milk composition have been supported by the pharmaceutical industry. The estimated three billion dollars/year market for bioreactors in USA [61] has fuelled the interest of the pharmaceutical industry. They quickly realized the potential capacity of synthesis and secretion of the mammary gland and several pharmaceutical products have already been synthesized in milk. The efficiency of the mammary gland as bioreactor is so high that production costs can be drastically reduced [28] even though the purification process is far from efficient. However, the post-translational ability is limited in some cases [11] and other organs with the ability of high protein synthesis and easy access to the recombinant product have been explored, for example the bladder and its secretory product, urine [29], or seminal plasma [49].

The mammary gland as bioreactor for therapeutic agents has been reviewed extensively in the last few years from several points of view [12, 26, 61, 65]. Little more information can be added, but it has to be mentioned that not only pharmaceutical proteins can be profitably produced in the mammary gland. Biologically active peptides can also be produced in a cost-effective way. Recently calcitonin, a peptide essential for the correct calcium metabolism and used in the treatment of osteoporosis, has been pro-

duced in transgenic rabbits. The transgene construct designed not only drove expression to the mammary gland, but also offered the necessary substrate for the effective endogenous amidation of the carboxy terminus, which took place in the mammary gland, producing a biologically active peptide [38].

The direct impact of biopharming in the dairy industry is low. Very few animals would provide enough raw product to cover the market demands and most of the research is focused on small ruminants as the species of choice [3]. However, an important indirect effect of biopharming that can be expected is a change in the consumer's perception of transgenic products in milk that could be envisioned as a source of human health care.

4. MODIFICATION THAT CAN BENEFIT THE DAIRY INDUSTRY

Biopharming industry views milk as a necessary side product of a marketable protein produced by transgenic modification. The dairy industry has to envision transgenic modification as the way to produce better milk. We cannot forget that milk is not only food, but also the raw material of a powerful industry that can benefit from any change that increases the manufacturing efficiency. Traditionally the price of milk has been based on the fat content, but somehow this concept is changing, and other components, such as proteins, are gaining preponderance.

Valuable modifications can be achieved in two ways, either quantitatively, by changing the proportion of milk components, or by means of a qualitative modification; that is, by adding other components, not present in the natural composition of milk, which will enhance its nutritional value. Milk has four major components: fat, protein, lactose and carrier, and even though it is possible to alter their proportions through genetic selection [16] there are clear limitations. Several experiments with transgenic mice have shown that it is possible to design a kind of milk that adapts better to a given industrial process or to the consumer demand, and to obtain it in a single generation.

5. QUANTITATIVE CHANGES IN MILK COMPOSITION THROUGH TRANSGENESIS

5.1. Changes in protein content of milk

The economical gain derived from increasing casein content in milk has been studied in detail [28] estimating the direct impact on the cheese industry. Caseins are the major proteins in milk and they develop an important functional activity. Casein micelles entrap fat and water of the milk during cheese manufacture, thus having a critical role in the cheese yield. The presence of extra κ-casein in transgenic mice [20, 21] resulted in significantly increased curd strength and a smaller micelle diameter. Even though no animal model has yet been produced, with the increase in α- and β-caseins, it can be assumed that these modifications would accelerate the speed of curd formation, its firmness and would also increase the calcium content [25].

It seems that the mammary gland has a limit in its capacity of protein synthesis. Any extra production is compensated by a decrease in the levels of endogenous milk proteins [37]. Based on these findings, another possible way to increase casein levels, still to be tested, should be the suppression of other proteins of limited interest. β-Lactoglobulin is the natural choice for this role. This protein, only present in the milk of ruminants, is the main allergen of bovine milk and its reduction would clearly improve human consumption. First it has been necessary to produce a mouse model with β-lactoglobulin expression [22] and then to design a strategy to silence its expression. As we mentioned before, transgenic

technology in livestock does not allow targeting of specific genes. In order to decrease or suppress the activity of a given gene, routes such as antisense RNA or ribozymes have to be used [52]. Ribozymes are able to cleave specific transcripts and we intend to use them for targeting of β-lactoglobulin. We have already generated transgenic mice, but we still have to test if the ribozymes secreted are able to specifically interfere with the β-lactoglobulin expression, at what level, and if this protein reduction involves an increase in the secretion of other endogenous proteins.

5.2. Altering the fat content

The producer's perception of fat content in milk is changing. The consumption of milk fat is diminishing. Fats of vegetal origin, considered as more healthy and produced at lower costs, are gradually but inexorably substituting animal fat. Further more, the mammary gland uses most of the energy consumed to produce fat [3], and a decrease in fat synthesis could be translated in a consistent reduction in feeding expenses [64]. Fat reduction in milk through genetic selection is difficult because of the high correlation ratios with proteins and it also has to be considered that altering milk fat composition could alter physical qualities of milk, such as the melting point [17], affecting the industrial transformation of dairy products. This implies that modifications of these characteristics would be restricted to certain niche markets [61].

It has been proposed that a decrease in the acetyl CoA expression might reduce de novo fat synthesis [4]. It is also possible to increase the expression of stearoyl-CoA desaturase, and in this way to reduce the presence of saturated fatty acids in milk, improving its dietetical quality for direct consumption. However, this modification would diminish the hardness of butter and could impair whipping of cream, and hence it has limited application.

5.3. Reducing lactose levels

Lactose is the major sugar present in milk and it is responsible for digestive disorders in a high proportion of the adult population [43] in which the levels of the intestinal enzyme lactase phlorizin hydrolase are reduced at weaning. This enzyme hydrolyses lactose in monosacharides. Several industrial approaches have been directed to the production of milk with reduced lactose content, such as treatment with lactases after harvest. This would not only benefit human consumption, in the dairy industry it would also result in an increased efficiency in cheese manufacturing [24].

Transgenic technology has approached this goal from different points of view. First, some experiments were focused on the production of low lactose milk. To achieve this, α-lactalbumine deficient mice were produced through homologous recombination [56], because this protein is one of the components of the lactose synthetase complex [40]. As a consequence of this genetic manipulation, mice produced milk with little or no lactose. However, this sugar plays a role in the osmotic regulation of the mammary gland, and, besides lower lactose levels, a negative side effect was observed, null mice produced little milk with high viscosity and were unable to sustain their progeny [55]. Ribozymes have also been used to reduce lactose level through the inactivation of α-lactalbumine. In this particular case, transgenic mice expressing the bovine α-lactalbumine were produced first, and then they were crossed with transgenic mice carrying a specific ribozyme, able to discern bovine α-lactalbumine from the endogenous murine protein. On the double transgenic animals, a consistent reduction of the protein targeted was observed, without effect on the endogenous protein [32].

The problems that have arisen while targeting α-lactalbumine expression are a good example of side effects when the mammary gland is used to secrete proteins that affect

animal health. Recently a controlled gene expression strategy has been designed to control the production of recombinant proteins in milk [54] based on a tetraciclyne transactivation. With this approach, transgenic animals, α-lactalbumine deficient, which produced highly viscous milk owing to a lack of lactose, were restored to their normal lactation. As we will mention shortly, lactose decrease in milk has been fully resolved, but the model proposed at Inra is a good solution when toxic agents have to be produced in the mammary gland.

The latest approach designed to reduce lactose levels in milk has been published recently, and it addresses the subject from an elegant and innovative point of view [27]. The authors propose to carry out in vivo the hydrolization that the dairy industry performs in vitro in order to adapt milk for non-tolerant human consumers. Lactose is hydrolysed by the ectopic expression of intestinal lactase in the mammary gland. In this way, in a single step the two problems are addressed, no impairment of milk production takes place, because the osmolar activity of lactose is preserved, and the concentration of this sugar is consistently reduced in the milk with no side effect on the suckling pups. The practical application of this experiment in cattle will require both the dairy industry and the consumers to recognize this strategy as a good alternative to expensive in vitro treatments to generate healthier milk.

6. QUALITATIVE CHANGES IN MILK COMPOSITION

Besides its intrinsic value, milk can also become a vehicle of other agents with different activities that enhance its properties, not only nutritional, but also functional. One example is lactoferrin, a whey protein of human milk with bacteriostatic effects that enhances iron absorption. This protein is present at very low levels in cow milk, and by increasing its presence, several goals can be achieved. Transgenic mice expressing human lactoferrin have been produced [48] to demonstrate that this protein increases iron absorption and protects neonates because it limits the free iron available in the intestinal tract, controlling bacterial multiplication. This approach would also allow the production of humanized milk, easier to transform into infant formula. With this idea in mind a transgenic bull carrying the human lactoferrin gene was produced [31].

The bacteriostatic effect can also be achieved with other proteins targeting the mammary gland itself, and hence reducing the incidence of mastitis. One example is human lysozyme as was demonstrated [35]. This protein not only had this antibacterial effect, it also favoured cheese yield owing to its association with caseins [34]. It is also possible to induce specific passive immunity in neonates with the secretion of specific antibodies. High titles of neutralizing antibodies have been obtained in milk of transgenic mice against specific viral agents [7, 53] targeting the expression of specific immunoglobulines into the mammary gland.

By secreting human proteins in cow milk, it is possible to humanize it and make it more adequate for human consumption. The inclusion of human lactoferrin, human lysozyme or human inmunoglobulines are an example but they also have an additional therapeutic benefit as was mentioned before. However, it is possible to substitute an animal protein by its human homology without apparent effect on the animal's physiological activity. At least this is what has occurred in mice lacking murine α-lactalbumine but expressing the human equivalent [55] which were able to lactate normally.

Milk can also be a vehicle of choice for nutraceuticals. Nutraceuticals or functional foods are foods or food components that have preventive or therapeutic effects against a variety of disorders. Familiar examples of this kind of product are *Lactobacillus acidophilus* and *bifidobacteria*. The expected

increasing global value of this market will reach $500 billion by 2010 [46]. This important economical niche is also being explored by biopharming industries. They have realized that these products might be marketed in a shorter time because food regulations do not imply the expensive clinical trials that therapeutic molecules have to fulfil. Several milk modifications we have mentioned above could fit into the definition of nutraceutical, such as calcitonin, lactoferrin or lysozyme. Also, the production through transgenesis of new molecules is being studied, such as prolactin, which enhances the immune defences and the growth of white cells [1].

7. CONCLUSIONS

Scaling up transgenic technology from the laboratory mouse to the dairy industry requires several factors. First, transgenic cattle have to be produced in a more efficient way. Technical advances published in the past 3 years suggest that this might be achieved in a short time. Second, the modifications proposed have to produce a clear added value to milk. We have presented a number of attractive strategies that allow a consistent improvement of milk, from both the quantitative and the qualitative points of view. New niches, such as nutraceuticals, will probably increase possible applications in the near future, and if the cost of making transgenics is reduced as a result of a more efficient technique, more research groups will address this matter. The possibility of homologous recombination and targeting of specific genes in cattle, now almost within reach, also opens new horizons. Third, and probably the most critical, the necessary fuel to make this a reality is to change the consumers' perception of genetically modified products as a source of food. Political and economical decisions are made based on public demands, and at the moment the consumers in Europe are far from willing to use transgenic products. In addition to the scientific and technical effort, it is necessary that we also adopt an informative role in order to be able to find genetically modified milk in the market in the next century.

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