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Review

Improving livestock for agriculture – technological progress from random transgenesis to precision genome editing heralds a new era

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Humans have a long history in shaping the genetic makeup of livestock to optimize production and meet growing human demands for food and other animal products. Until recently, this has only been possible through traditional breeding and selection, which is a painstakingly slow process of accumulating incremental gains over a long period. The development of transgenic livestock technology offers a more direct approach with the possibility for making genetic improvements with greater impact and within a single generation. However, initially the technology was hampered by technical difficulties and limitations, which have now largely been overcome by progressive improvements over the past 30 years. Particularly, the advent of genome editing in combination with homologous recombination has added a new level of efficiency and precision that holds much promise for the genetic improvement of livestock using the increasing knowledge of the phenotypic impact of genetic sequence variants. So far not a single line of transgenic livestock has gained approval for commercialization. The step change to genome-edited livestock with precise sequence changes may accelerate the path to market, provided applications of this new technology for agriculture can deliver, in addition to economic incentives for producers, also compelling benefits for animals, consumers, and the environment.

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1 Introduction

Over the past 30 years, the capability to genetically modify livestock has dramatically changed with the successive development of improved techniques. The dominant technique that was used early on relied on the direct injection of exogenous DNA into one-cell embryos. This essentially restricted the range of modifications to the addition of a gene construct without any control over when and where the new gene was integrated into the genome. This limitation was initially overcome with the development of a cell-mediated technology for livestock transgenesis. Although now possible, site-specific approaches using homologous recombination (HR) to target endogenous genes and integrate gene constructs into defined chromosomal loci have proved to be highly inefficient with this approach due to reliance on primary cells [1]. The next big technological advancement, genome editing with site-specific nucleases, removed this efficiency barrier by providing the ability to readily disrupt genes and introduce specific mutations. Here, we will review the improvements in the enabling technologies to genetically modify livestock as illustrated by pig, sheep,

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Abbreviations: BLG, beta-lactoglobulin; **Cas,** CRISPR-associated nuclease; **CRISPR,** clustered, regularly interspaced short palindromic repeat; **ESC,** embryonic stem cell; **FDA,** Food and Drug Administration; **GH,** growth hormone; **GRF,** growth hormone releasing factor; **HR,** homologous recombination; **IGF,** insulin-like growth factor; **indel,** small insertion or deletion; **MI,** microinjection; **NHEJ,** non-homologous end joining; **PrP,** prion protein; **PUFA,** poly-unsaturated fatty acid; **RELA,** v-rel avian reticuloendotheliosis viral oncogene homolog A; **SCNT,** somatic cell nuclear transfer; **SCD,** stearoyl-CoA desaturase; **shRNA,** short hairpin RNA; **TALEN,** transcription activator-like effector nuclease; **UFA,** unsaturated fatty acid; **ZFN,** zinc-finger nuclease

Table 1. GM livestock with relevance for agriculture produced by different technologies

Technology	Trait	Species	Modification[a)]	Reference
Microinjection	Improved meat production	Pig	GH	[5, 7, 8]
		Pig	GRF	[4, 7]
		Pig	IGF	[6]
		Sheep	GH	[9, 11]
		Sheep	GRF	[10]
	Increase wool production	Sheep	IGF	[13]
	Improved wool	Sheep	IF	[14]
	Improved milk proteins	Pig	αLac	[15]
		Cattle	LF	[17]
		Goat	LF	[18]
		Goat	LZ	[16]
	Improved milk fat	Pig	FAD2	[22]
		Goat	SCD	[21]
	Disease resistance	Pig	mAB	[23, 24]
		Sheep	mAB	[23]
	Decreased environmental impact	Pig	Phytase	[25]
Cell-mediated by SCNT, random	Improved milk proteins	Cattle	Casein	[32]
		Cattle	BLG miRNA	[43]
		Cattle	αLac	[42]
		Cattle	LF	[41]
		Cattle	LZ	[35]
		Pig	LZ	[39, 40]
	Improved animal derived foods	Pig	Fat-1	[45, 46]
		Cattle	Fat-1	[47]
		Sheep	Fat-1	[48]
	Improved meat production	Cattle	MSTN shRNA	[107]
	Disease resistance	Cattle	LSS	[34]
		Pig	FMD shRNA	[49]
		Goat	PrP shRNA	[51]
		Cattle	PrP shRNA	[52]
Cell-mediated by SCNT, targeted	Disease resistance	Sheep	PrP	[68]
		Goat	Prp	[65, 66]
		Cattle	Prp	[64]
	Improved meat production	Goat	MSTN	[69]
Genome editing, random indels	Improved milk proteins	Cattle	BLG	[74]
	Improved meat production	Cattle	MSTN	[75, 79]
		Sheep	MSTN	[79, 88]
		Goat	MSTN	[86]
	Disease resistance	Pig	RELA	[78]
Genome editing, precise mutations	Disease resistance	Cattle	βCN, LSS	[81]
		Cattle	βCN, LZ	[82]

a) αLac, alpha-lactalbumin; βCN, beta-casein; IF, intermediate filament keratin; LF, lactoferrin; LSS, lysostaphin; LZ, lysozyme; MSTN, myostatin; all other abbreviations as defined in the main text.

goat, and cattle models with improved agricultural traits that have been developed over the past three decades (Table 1). In addition, implications of the changes in technology on the regulation and public perception of such animals will be discussed.

2 The beginnings – pronuclear microinjection (MI) of DNA

The development of transgenic mouse technology made it possible to manipulate the mammalian genome and study the function of individual genes through gain and loss of function approaches in the context of live mouse models. The demonstration in 1980 that the direct MI of

an exogenous gene construct into the pro-nucleus of a recently fertilized mouse zygote can efficiently generate transgenic mouse models provided a relatively simple method for mammalian transgenesis [2]. The ensuing widespread use of MI by scientists around the world resulted in a plethora of mouse models that had an immense impact on biomedical research. However, transfer of the technology to livestock species proved challenging with greater technical difficulties and lower efficiencies in producing transgenic offspring than in the mouse system [3]. In the absence of other alternatives, MI was for many years the only available method for developing transgenic livestock and the enabling technology for the first wave of transgenic livestock models (Table 1). These early studies had a focus on increasing meat production by overexpressing growth factors such as growth hormone (GH), growth hormone releasing factor (GRF), and insulin-like growth factor (IGF) to enhance muscle growth in pigs [4–8] and sheep [9–11]. Technical difficulties to accurately control the expression of these highly bioactive factors resulted in high systemic levels of these growth factors and were associated with adverse health effects [8, 12]. Conditional expression strategies were able to resolve these problems [5]. Beside the focus on meat, additional aims in sheep were increased wool production and improved fiber with better processing and wearing qualities [9, 13, 14].

Another major area of interest was the improvement of milk composition with the aim to increase animal production efficiencies or enhanced human nutrition. While the overexpression of the milk protein alpha-lactalbumin in the milk of pigs had a marked effect on the survival rate of suckling piglets and thus, pig production [15], the introduction of human variants of the antimicrobial proteins lactoferrin and lysozyme enhanced the health benefits of dairy milk as a human food by aiding the intrinsic defense mechanisms against pathogenic microorganisms [16–18]. Increased levels of the antimicrobial proteins also helped to increase the shelf life of milk and protect lactating animals against mastitis-causing pathogens [19, 20]. In addition, milkfat has been a target for improvement of milk because it contains high levels of unsaturated fatty acids (UFAs) that have been widely associated with cardiovascular and coronary heart disease. Overexpression of a mammalian enzyme involved in converting saturated FAs into mono-UFAs, stearoyl-CoA desaturase (SCD), improved the ratio of unsaturated to saturated FAs in milkfat although only transiently due to an apparent instability of the mRNA transcribed from the transgene [21]. This concept was taken even further with the introduction of a transgene for the expression of the Δ12 FA desaturase (FAD2) from spinach, which is involved in the synthesis of the essential poly-UFA (PUFA) linoleic acid. In transgenic pigs, the linoleic acid content of adipose tissue was increased by 20% [22]. It validated the feasibility of modifying livestock with the ability to endogenously synthesize essential PUFAs and the prospect of producing animal derived foods rich in PUFAs that could deliver human health benefits such as reduced risk for coronary heart disease. But also the health and welfare of livestock has been a primary target. Two pioneering studies demonstrated the feasibility of expressing mABs in the serum of transgenic pigs to potentially provide enhanced in vivo immunity against specific pathogens [23, 24].

In addition, the versatility of the technology provided sufficient scope to test novel strategies for mitigating the adverse environmental effects from intensive farming systems. Due to the introduction of a transgene for the expression of a bacterial phytase in the salivary gland, transgenic pigs can utilize the otherwise unavailable phosphate by hydrolyzing the normally indigestible phytate contained in their food [25]. This resulted in the remarkable reduction of phosphate in their manure by up to 75%, which could greatly reduce phosphate leaching and eutrophication of waterways and aquifers from intensive pig farming activities.

3 Increased control – cell-mediated transgenesis

The discovery of murine embryonic stem cells (ESCs) signified a remarkable step-change in the technology with the ability to make precise changes at specific genomic sites [26]. The unique characteristics of these pluripotent cells, being able to give rise to all differentiated cell types of an adult animal, indefinite growth in culture and high HR efficiency, provided the avenue for a more sophisticated cell-mediated transgenesis method with essentially unlimited possibilities to precisely modify the mouse genome. The genome of ESCs is readily amenable to precise changes such as the integration of a single transgene copy into a predefined site or the functional disruption of a specific endogenous gene. Due to their pluripotent nature, ESCs can contribute to all tissues, including the germline, following their injection into or aggregation with a host embryo. By breeding from the resulting chimeric mice live transgenic mice can then be produced that are entirely derived from fully characterized GM ESCs. Despite great international efforts such cells have so far not been isolated from livestock species and this major technological advancement in the mouse system remained unavailable for the genetic modification of livestock.

An alternative approach for a cell-mediated transgenesis method that could be applied in farm animals finally emerged in 1996 with the arrival of the clone sheep Dolly, the first mammal produced by somatic cell nuclear transfer (SCNT) [27]. Shortly thereafter, it was shown that somatic cells, which had previously been subjected to genetic manipulations could indeed serve as donors for

the generation of live transgenic offspring by SCNT [28]. Highlighting the great potential of the new technique, the authors reported that their production of transgenic sheep by SCNT required fewer than half the animals needed for pronuclear MI. Due to the greater efficiencies and the potential to perform a wide range of modifications, including site-specific changes such as knockout and knockin, SCNT quickly replaced MI as the preferred technology to generate GM livestock. Still, SCNT is not a livestock equivalent of mouse ESC technology and is plagued by a number of substantial limitations. During SCNT the gene expression pattern of a somatic cell needs to be reprogrammed to one that is comparable to that of a zygote. However, this process is often incomplete or incorrect which compromises the developmental potential of the embryo and is the main cause for the relatively low efficiency in producing healthy cloned offspring [29]. Epigenetic changes that occur during reprogramming also affect transgenes introduced into somatic cells and in particular in conjunction with bacterial antibiotic selection markers may lead to variegated transgene expression [30]. Moreover, the limited proliferative capacity of primary somatic cells constrains the scope for more complex, site-specific modifications and often requires an intermediate step to rejuvenate the primary cells by re-deriving cell lines with restored growth potential from cloned fetuses [31]. The resulting prolonged time in culture and multiple rounds of cloning further increases the risk for the accumulation of genetic and epigenetic abnormalities.

3.1 Random transgene insertions

However, it was primarily the greater efficiency of SCNT in generating transgenic founder animals compared to MI that led to the development of a new generation of transgenic livestock models for agricultural applications, with most studies being content with the random insertion of the transgene (Table 1). The efficiency gain provided sufficient scope to transfer old concepts on improving the composition of cows' milk, previously only evaluated in mouse models, into dairy cattle as the ultimate target species. Aimed at increasing the amount of casein in cows' milk, Brophy et al. introduced additional copies of beta- and kappa-casein into bovine fibroblasts, which were subsequently used to produce live calves by SCNT [32]. Analysis of the milk, first in hormonally induced calves and later in naturally-lactating animals [33], showed a slight increase in beta-casein and marked upregulation of kappa-casein. The total protein concentration was only slightly increased, which suggests that milk protein production is tightly regulated and that the transgenic casein variants were at least partially expressed at the expense of endogenous milk proteins. In a project intended to lowering disease-associated production losses and improving animal welfare by targeting mastitis infections caused by *Staphylococcus aureus*, bovine fibroblasts were transfected with the gene for the peptidoglycan hydrolase lysostaphin and used as SCNT donor cells to generate live offspring [34]. Following an *S. aureus* challenge in a comparative assay, wild type cows displayed symptoms of mastitis while expression of as little as 3 μg/mL of lysostaphin provided protection in transgenic animals. The compositional differences between the milk of humans and livestock species sparked a number of projects directed at humanizing the milk of dairy animals. Re-visiting the idea of enhancing the concentration of antimicrobial proteins, found at much higher levels in human compared to dairy milk, with an SCNT approach, Yang et al. introduced gene copies for human lysozyme into bovine fibroblasts and produced cows from these donor cells [35]. Lysozyme expression was about one tenth of the levels achieved with the earlier MI-generated goat model. While beneficial effects from the consumption of the lysozyme enriched goat milk, including improved gastrointestinal health and clearance of diarrhea causing infections, have been well documented [36–38], this still needs to be demonstrated for the cows' milk. Similarly, human lysozyme has also been overexpressed in pigs' milk in transgenic animals derived from SCNT donor cells possessing copies of the human lysozyme gene [39]. Milk produced by the transgenic pigs was shown to inhibit the growth of *Escherichia coli* in the duodenum and positively influence intestinal morphology in suckling piglets when comparing the effects of transgenic and non-transgenic milk [40]. Another early transgenic bovine model generated by MI aimed at the overexpression of the antimicrobial milk protein lactoferrin was replicated using the contemporary SCNT technology [41]. Transgenic cattle generated from fibroblasts microinjected with a bacterial artificial chromosome construct carrying the human lactoferrin gene locus expressed high levels of lactoferrin in milk, comparable to the older, MI-generated cattle line. This makes these animals foremostly very attractive "bioreactors" for extraction of the protein from milk, although the milk's high lactoferrin content is also expected to provide health benefits for human consumption.

Another attempt to humanize milk was undertaken by expressing human alpha-lactalbumin in cloned cattle. Its overexpression had no apparent effect on the amounts of the endogenous milk proteins compared to normal milk and thus ought to provide improved nutritional value for the transgenic milk [42]. Transgenic livestock producing humanized designer milk are not limited to the portrayed examples of animals that additionally produce human proteins in their milk. Humanizing milk may also entail the removal of undesired proteins such beta-lactoglobulin (BLG), which causes allergies, especially in infants. A transgenic calf, generated by SCNT from fibroblasts engineered for the lactation-specific expression of microRNAs (miRNAs) with target specificity for BLG, no longer

expressed the allergenic protein in its milk which hints at the potential of this milk as a source for hypoallergenic dairy products, particularly infant formula [43].

Diets in many western countries are rich in animal-meats and typically have a high ratio of n-6:n-3 PUFAs, which has been attributed to a variety of serious health problems such as cardiovascular diseases, cancer, arthritis, and diabetes [44]. A major reason that farm animals' meat is rich in n-6 and low in the health promoting n-3 PUFAs is their inability to convert n-6 to n-3 PUFAs. Analogous to the earlier study by Saeki et al. [22], SCNT-mediated transgenesis has been employed to improve the essential FA composition of meat by introducing a FA desaturase that can convert n-6 to the more beneficial n-3 PUFAs. Constitutive expression of the humanized form of the *Caenorhabditis elegans* fat-1 desaturase (Fat-1) in pigs [45, 46], cattle [47], and sheep [48] resulted in significantly higher n-3:n-6 FA ratios compared to wild type animals.

SCNT cloning has also been applied to generate transgenic animals with enhanced disease resistance to improve animal welfare, food safety, and risk to human health. Foot and mouth disease is one of the most severe diseases affecting productivity in livestock. Vaccinations against the disease-causing virus have shown promise but they are afflicted with problems such as delayed immunity or the inability to combat mutated variants of the virus. Expression of designer short hairpin RNAs (shRNAs) in cloned transgenic animals that target crucial viral functions to disrupt virus propagation has been proposed as an alternative to vaccination. Cloned pigs expressing shRNAs against viral proteins exhibited delayed disease symptoms compared to non-transgenic control animals upon viral challenge and post-mortem analyses suggested that shRNA expression could prevent virus transmission [49]. Similarly, shRNAs have also been shown to provide protection against foot and mouth disease in cells derived from cloned bovine fetuses [50], although live animals have yet to be produced. RNA interference and SCNT were also combined in projects intending to prevent transmissible spongiform encephalopathies caused by misfolded variants of the prion protein (PrP), so-called prions. A goat fetus, generated from donor cells with lentivirally-introduced copies of shRNAs targeting PrP showed a significant reduction of PrP [51], but animals have yet to be generated. Knockdown by RNA interference was also attempted in cattle, where expression of PrP-targeting shRNAs in a cloned calf resulted in only marginal reduction of the PrP protein [52].

Recently, transposon systems have emerged as an extremely efficient option for cell-mediated gene transfer in livestock species [53–55]. The system relies on the activity of a transposase which can integrate transgenes that are flanked by compatible inverted terminal repeats into random sites; although some transposases show preferences for specific sequence motifs. The copy and paste mechanism results in the integration of only a monomeric transgene copy. However, this may happen at multiple loci and can lead to complications with segregating insertion sites from transgenic founder animals that were generated by SCNT using cells with multiple transgene insertion sites. Moreover, the efficiency gains from the applications of transposons for cell-mediated transgenesis could also be transferred to the gene transfer in embryos [53, 56, 57]. In combination with direct cytoplasmic injection transposon technology provides for the first time an efficient route to generate transgenic livestock by MI. Yet, the technology still awaits its first application to improve a trait with relevance for agriculture.

3.2 Recombinase-mediated insertions into pre-selected sites

For improved control over the performance of randomly inserted transgenes, recombinase-mediated targeted insertion enables the repeated use of validated, so-called safe harbor insertion sites. In a first step, genomic loci are tagged with the insertion of an exchange cassette that is flanked by non-homolgous recombination motifs. Because of its efficiency in integrating monomeric copies transposon technology is particularly well suited for the tagging. Following the verification as a transcriptionally permissive locus, in a process called recombinase-mediated cassette exchange, the cognate recombinase can then be used to insert a gene of interest, which needs to be flanked by the same recombination recognition sites, into the tagged locus via an exchange of the content of the integrated cassette. Efficient cassette exchange systems have been developed for the Cre and flippase recombinases [58]. So far, the flippase system has only been validated in an immortalized pig cell line [59] whereas Cre-mediated recombination has been applied for the targeted insertion of transgenes in bovine somatic cells [60] and was shown in pigs to be compatible with the generation of fetuses and pigs following SCNT [56, 61].

3.3 Gene targeting by homologous recombination

While the overexpression models described above benefited from the efficiency of generating transgenic animals from cells that had been fully verified for the presence of the transgene, SCNT technology also offers the ability for site-specific modifications by HR. However, the short lifespan and low HR efficiencies of primary somatic cells rendered gene targeting in livestock species extremely challenging [62]. This has greatly limited the number of livestock models with site-specific modifications, almost all of which have been produced for biomedical or biopharmaceutical purposes, as disease models or as potential donors for xenotransplantation due to greater economic incentive, ethical justification, and public acceptance for biomedical applications [1].

The only transgenic livestock models with an agriculturally relevant phenotype developed to date by HR targeting were disruptions of the PrP gene, generating livestock resistant to the neurodegenerative prion diseases (Table 1). PrP gene knockouts have been accomplished in cattle using homozygous PrP knockout cells as donors for nuclear transfer [63, 64], in goats using heterozygous PrP knockout cells as SCNT donors and then producing homozygous PrP knockout goats through breeding [65, 66] or using homozygous PrP knockout cells for SCNT [67] and in sheep, although PrP–/+ lambs did not survive beyond day 12 [68]. HR was also attempted to knockout myostatin, a negative regulator of muscle growth. A targeted goat fibroblast cell line was used as donor for the generation of a transgenic kid that died shortly after birth [69]. Western blot analysis revealed only trace amounts of myostatin, but whether this reduction would render the transgenic goats hyper-muscular remains to be demonstrated. Monoallelic myostatin knockouts via gene targeting were also accomplished in sheep cells [70, 71] but SCNT to produce live animals has yet to be undertaken. These examples also illustrate the intrinsic difficulties associated with the generation of gene targeted animals by SCNT. The low HR efficiencies of primary somatic cells in combination with their restricted proliferative capacity limits the isolation of targeted cell clones to a select few which do not necessarily have the developmental competency to give rise to healthy live transgenic animals. Although possible with the application of stringent selection strategies, HR was inefficient and the percentage of selected cell clones with one correctly targeted allele often well below 10% [1]. Thus, on its own, SCNT-based gene targeting proved to be too inefficient to make the introduction of site-specific changes into livestock genomes a routine practice.

4 Combining precision with efficiency – genome editing

The remarkable development of genome editing in the last few years is set to revolutionize the field due to the prospect that for the first time large animal genomes can be modified with sophistication and efficiency so far only achievable in the mouse system. The introduction of site-specific DNA double strand breaks by chimeric designer nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) that combine a customizable DNA binding domain with the catalytic domain of the restriction endonuclease FokI can induce gene disruptions or trigger homology-driven genome modifications with unprecedented ease [72, 73]. Moreover, genome editing technology allows for the introduction of mutations without leaving any technology-associated footprint and thus results in modifications of the genome that are indistinguishable from natural mutations.

4.1 Unspecified, site-specific mutations

In an immense improvement of the established SCNT-mediated transgenesis capabilities, DNA- or RNA-encoded genome editing nucleases can be transfected into primary somatic cells to induce site-specific mutations at such efficiencies that drug selection is no longer required. Typically, targeting can be achieved in 1–50% of all transfected cells, including significant proportions of biallelically modified cells [73]. In the absence of an exogenous repair template, nuclease-stimulated double strand breaks are repaired by non-homologous end joining (NHEJ). This endogenous repair mechanism is error prone and does not always result in the faithful repair of the double strand break but sometimes produces small insertions and deletions (indels) or point mutations. Thus, designer nucleases can be utilized to produce randomly generated modifications, e.g. knockouts, at the targeted locus (Table 1). This technology was used to improve the composition of cows' milk by preventing the expression of the allergenic milk protein BLG. NHEJ was harnessed to modify the bovine gene for BLG following ZFN cleavage near the start codon of the gene. Cells with heterozygous biallelic modifications were used as donors for the generation of live offspring. However, the two allelic variants present in the only surviving calf had small in-frame deletions which did not disrupt the BLG reading frame and thus modified the gene but did not produce a knockout phenotype [74]. In a similar approach, ZFN cleavage-triggered NHEJ yielded biallelic modifications of the bovine myostatin gene. Myostatin protein levels in SCNT-produced calves were reduced by about 50% and the animals displayed a double muscled phenotype at the age of one month [75]. Although the SCNT-mediated genome editing approach has the advantage that the introduced mutation can be fully characterized and allows that only transgenic animals with desirable, even biallelic mutations are generated, this approach suffers from a major downside. SCNT is notoriously encumbered by low production efficiencies of viable cloned offspring due to a high incidence of developmental abnormalities. The direct injection of genome editing tools into one cell embryos circumvents the inefficiencies of producing transgenic animals by SCNT and offers a compelling alternative. This is further aided by only requiring simple cytoplasmic injections instead of pronuclear injections, which are technically difficult in livestock because the pronuclei are often totally obscured in livestock species. First validated in rats with ZFNs [76], the generation of bovine embryos that were successfully genome edited at the myostatin locus and the ACAN (aggrecan) gene, including biallelical modifications, following cytoplasmic MI of TALENs into zygotes demonstrated the potential of this technique to produce transgenic livestock with high efficiencies [77]. The same study described the modification of the v-rel avian reticuloendotheliosis viral oncogene homolog A (RELA) locus

in pig embryos, which was prompted by the association of allelic variants of the RELA gene with tolerance against African Swine Fever Virus infections in pigs. Progression of this line of experiments subsequently produced the first live pigs derived from genome-edited embryos, including piglets with heterozygous (5% of born) and homozygous (4% of born) biallelic modifications of the RELA gene [78]. Subsequently, the successful application of direct TALEN injection into zygotes was extended to cattle and sheep with the generation of targeted mutations in the myostatin gene [79].

4.2 Precision mutagenesis

While the efficient disruption of endogenous genes through the induction of indels has already been an enormous improvement, the ability of the genome editing technology to trigger homology-driven repair of double-strand breaks in the presence of an exogenous repair template is likely to provide an even greater impact in future applications. Although farm animals, modified by defined mutations specified on oligonucleotides as homologous repair templates have yet to be reported, co-transfections of TALENs and "repair"' oligonucleotides have yielded large numbers of targeted dilution clones (3–67%) with a large proportion (up to 59%) edited on both alleles [80]. These cells can be readily used as SCNT donors for the production of animals with beneficial agricultural traits such as hyper-muscularity or polled (hornless) in cattle, increased muscle growth or fecundity in sheep and disease resistance in pigs. In contrast to precision editing with oligonucleotides, the application of genome editing with much longer homologous targeting vectors as repair templates for the introgression of whole genes has already been exemplified with the production of transgenic cattle (Table 1). ZFN-assisted gene targeting was used to knockin the gene for lysostaphin [81] and human lysozyme [82] into the beta-casein locus of primary bovine cells. Transgenic cattle produced via SCNT were shown to secrete lysostaphin in their milk and in vitro assays suggest that this can provide protection against *S. aureus* infections. Similarly, cattle generated from lysozyme knockin cells produced enzymatically active human lysozyme in their milk, which suggests increased resistance toward mastitis infections caused by *S. aureus*.

As if the novel capabilities offered by ZFNs and TALENs were not enough, a new genome editing tool emerged recently that promises even greater simplicity, flexibility, and efficiency and all at a lower cost. This latest tool is a RNA-guided nuclease system referred to as clustered, regularly interspaced short palindromic repeat (CRISPR) – CRISPR-associated nuclease 9 (Cas9) [83]. Contrary to ZFNs and TALENs, which need protein engineering to customize their DNA binding properties, the CRISPR-Cas9 system uses a universal monomeric nuclease (Cas9) that is guided by sequence complementarity of a small, so-called guide RNA, to its specific target site where it introduces a DNA double strand break. This makes it very simple to design CRISPR/Cas9 enzymes with different target specificity, essentially by just including a different oligonucleotide in the guide RNA expression construct specifying a different target site. At the same time, it greatly facilitates the simultaneous targeting of multiple targets. With the CRISPR-Cas9 system, this only requires the addition of a few more of the small guide RNAs and the Cas9 nuclease activity can be directed to different target sites in the genome. The extraordinary multiplexing capability of this system has recently been demonstrated in spectacular fashion by the simultaneous modification of all alleles of five different genes in mouse ES cells [84], which was quickly followed by the first reports of multiplexing applications targeting multiple genes in pigs and goats [85–87]. The breath-taking pace of the developments of the CRISPR-Cas9 technology has so far only allowed sufficient time for the publication of the very first reports on agricultural applications of the new technology platform. In goat primary cells, mono- and biallelic knockouts of the genes for myostatin, PrP, nucleoporin 155, and BLG were efficiently induced with CRISPR-Cas-mediated editing. SCNT with two independent cell clones that were confirmed to carry biallelic myostatin disruptions resulted in the generation of three myostatin knockout goats [86]. Direct injection of Cas9 mRNA and myostatin-specific guide RNA into sheep embryos produced two lambs with two and five differently edited alleles. Both lambs, still had copies of the wild type allele and were classified by the authors as monoallelic mutants [88]. Considering the advantages of the CRISPR-Cas9 system, the number of studies can be expected to quickly increase in the near future. Yet, there are already rumors spreading about the imminent release of new editors with the potential to supersede the known technology platforms.

The high efficiency of the CRISPR/Cas9 system comes at a price. Potent nuclease activity, in combination with only a short target recognition sequence and tolerance for mismatches, creates an increased potential for the introduction of double strand breaks at off target sites that can result in the introduction of unintended mutations somewhere else in the genome [89]. One strategy to reduce off target effects has been to inactivate one of the two Cas9 nuclease domains responsible for cutting each of the two DNA strands so the mutant enzyme can only introduce a nick into the target site instead of a double strand break. Any off target nicking activity remains relatively inconsequential as these are efficiently repaired with high fidelity by base excision. The introduction of a targeted double strand break now requires the synergistic co-localization of a pair of such nickases to introduce nicks with a small offset in opposing DNA strands, essentially generating a double strand break, which greatly increases the overall specificity [90, 91]. Still, the nicking activity of an individ-

ual monomer may be able to induce off target effects. To further increase the stringency, catalytically inactive forms of Cas9 were fused with the dimerization-dependent FokI nuclease domain. These RNA guided FokI nucleases, like ZFNs and TALENs, require not only the coordinated binding of a pair of monomers but also the dimerization of the FokI domain to gain nuclease activity and the ability to induce genome edits [92, 93]. While these improved CRISPR-Cas9 systems have not yet been applied in livestock species, a nickase strategy with a converted ZFN has been assessed for its knockin ability in cattle. The ZFNickase was generated by pairing a functional ZFN with a ZFN containing an inactive FokI domain. Upon dimerization, the functional FokI of this ZF protein can only introduce a nick at the target site. This was shown to be sufficient to enable the knockin of the lysostaphin gene into the bovine beta-casein locus, albeit with lower efficiency than the equivalent ZFNs [81].

5 The final hurdle – jumping out of the research realm and onto the farm

The ability to genetically modify animals was actually developed a few years before it was possible to generate transgenic plants. Despite this later start, transgenic plants quickly surpassed animals with the first GM crop, the FlavrSavr tomato, gaining market approval in 1994. Since then, transgenic plants have seen unprecedented adoption rates with transgenic crops now being grown by 27 countries on over 175 million hectares, translating into an over 100-fold increase in the global cultivation area of biotech crops between 1996 and 2013 [94]. The story could not be more different for transgenic animals. To date, no GM animal has been approved for entry into the food supply. While comparatively long reproductive cycles of livestock and animal welfare concerns are unique factors that do not apply to arable crops, regulatory uncertainty, lack of industry support, and interference at the political level are crucial determinants in preventing the commercialization of GM animal food products. This may be best exemplified by the so far unsuccessful attempts to gain market approval for a growth-enhanced salmon which was first generated in 1989. Despite initiating discussions with the regulatory authorities in 1993 and formally entering the regulatory pathway of the US Food and Drug Administration (FDA) as early as 1993, the process was riddled with delays and roadblocks [95]. The FDA concluded from their scientific evaluation in 2010 and 2012 that the GM salmon is safe to eat and unlikely to cause significant effects on the environment under the proposed production system. Two years on without further progress, market approval remains an elusive goal. Similarly, the Enviropig, which was developed in 1999, spent years in the maze of the North American regulatory system without ever reaching the finish line when finally the financial support for this project came to an end in 2012 (Schmidt, S., Postmedia News, http://www.canada.com/technology/Genetically+engineered+pigs+killed+after+funding+ends/6819844/story.html).

Although the European regulators pioneered the approval of ATryn, the first GM animal-produced human drug, two years ahead of its approval by the US FDA [96, 97], to date the EU regulatory pathway remains also untested for food products from GM animals. Contrary to the US FDA regulating such animals and their products as "investigative new animal drugs" with a focus on the product, the EU assessment is based on the comparison of the GM animal with its conventional counterpart, with a strong focus on the process used to generate the GM animal [98]. The obligation for reaching consensus decisions between the different EU member states has essentially blocked the regulatory process and only four GM crops have been approved by the EU since 1996. This is despite the fact that there is now overwhelming scientific evidence that GM technology per se poses no greater risks than conventional breeding technologies (European Commission, A decade of EU-funded GMO research (2001-2010); http://ec.europa.eu/research/biosociety/pdf/a_decade_of_eu-funded_gmo_research.pdf). Thus, supposedly science-based regulatory processes have increasingly become marred by political interference both in the USA and EU [99, 100]. The reluctance toward GM technology, however, comes at a cost. By not using GM technology, we risk missing out on environmental and animal and human welfare benefits [101]. While this has been largely ignored and can be afforded by the more affluent nations, it is already causing real suffering in other parts of the world [102].

At the same time and probably unnoticed by the public, genetic modification technology has seen breath-taking developments in recent years with capabilities for the rapid improvement of livestock. As an alternative to years of backcrosses over at least five generations, genome editing with designer nucleases enables the almost instant introgression of desirable traits into the genetic background of elite animal. Thus, the technology has the potential to provide a revolutionary solution to the inefficiencies of current breeding and selection systems that rely on the identification of not the best but the best available combination of allelic variants in individuals using whole genome selection. Particularly in combination with genomic selection of embryos could genome editing boost genetic gain by overcoming some of the restrictions caused by the absence of known beneficial alleles in the available gene pool, non-segregation of closely linked desirable and unwanted traits and the presence of detrimental alleles such as recessive mutations causing embryonic lethal phenotypes. Thus, the multiplexing capabilities of genome editing have great potential to directly introgress beneficial and eliminate detrimental alleles to greatly improve or rescue the best embryos iden-

tified by genome selection. Moreover, genomic selection uses the association of sequence variants, so called single nucleotide polymorphisms, with phenotypic traits. However, the causative variants with the greatest impact on important economic traits are often completely unknown. Introgression of specific single nucleotide polymorphisms by genome editing provides a tool to validate their impact and generates information that will be invaluable to improve genomic selection systems. This potential has not only excited scientists around the world but has also elicited the interest of the industry. Rapid progress of the technology, particularly with the more commercially advanced plant applications, has urged regulators into discussions on whether and how this genome editing technology should be regulated. Early indications have emerged from several regulators that support the view that the introduction of precise mutations via genome editing may not need regulatory oversight [103, 104].

6 Concluding remarks

Our increasing knowledge of the relationship between allelic sequence variations and phenotypic traits from whole genome SNP-chip and whole genome sequence analysis of large numbers of livestock animals is paralleled by improved technical capabilities to directly introgress specific allelic gene variants into livestock genomes. Bringing these developments together will allow for a radically new breeding approach to maximize genetic improvement that is no longer limited to random chance combinations but can unify the best allelic variants in individual animals. Due to its precision, genome editing has a very low risk profile and results in fewer, more controlled modifications compared with generally accepted technologies such as conventional breeding. However, genome editing is a relatively new technology and its regulatory status still needs to be clarified. But irrespective of whether genome editing might finally be classified as an unregulated activity, opponents of genetic modification are unlikely to change their anti-technology stance. Although they may represent only a small minority, due to the inference of representing the opinion of the general public, they command an disproportionally strong influence. The assumed prevalence of an unwaveringly negative public perception, despite some evidence for a much more measured public response (European Commission: Framework 6, Final report: Do European consumers buy GM food? http://www.kcl.ac.uk/medicine/research/divisions/dns/projects/consumerchoice/index.aspx) [105, 106], will remain a major concern for food producers, retailers and politicians. However, no animals and animal products are likely to enter the market without industry/political support for the technology. Conversely, without any products available, the public has no opportunity to gain direct experience with such products and true consumer attitudes will remain unknown. Hiding behind this chicken and egg dilemma and sacrificing a technology, which could substantially contribute to solutions to securing food security for a rapidly growing human population under constrains of decreasing resources and a changing world climate, is becoming increasingly irresponsible. Probably for the first time humanity faces truly global challenges in increasing environmentally sustainable food production, which urgently require global initiatives to break the current deadlock. Ultimately, this will require the development of genome-edited livestock that have been demonstrated to be safe and provide clear benefits to consumers, the animals and the environment as well as offering sufficient incentives for producers to use these animals.

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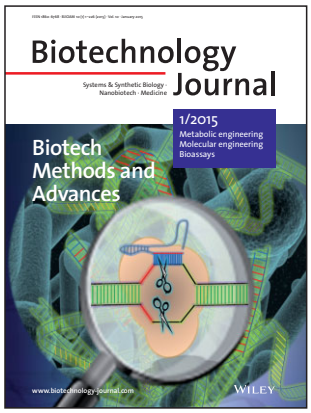

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Research Article

Fluorescence techniques used to measure interactions between hydroxyapatite nanoparticles and epidermal growth factor receptors

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http://dx.doi.org/10.1002/biot.201400404

Research Article

(+)-Valencene production in *Nicotiana benthamiana* is increased by down-regulation of competing pathways

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Research Article

High-throughput nucleoside phosphate monitoring in mammalian cell fed-batch cultivation using quantitative matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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http://dx.doi.org/10.1002/biot.201400292

Research Article

Glycoarrays with engineered phages displaying structurally diverse oligosaccharides enable high-throughput detection of glycan–protein interactions

Eda Çelik, Anne A. Ollis, Yi Lasanajak, Adam C. Fisher, Göksu Gür, David F. Smith and Matthew P. DeLisa

http://dx.doi.org/10.1002/biot.201400354

Research Article

Xylan degradation improved by a combination of monolithic columns bearing immobilized recombinant β-xylosidase from *Aspergillus awamori* X-100 and Grindamyl H121 β-xylanase

Maria V. Volokitina, Kirill S. Bobrov, Kathleen Piens, Elena V. Eneyskaya, Tatiana B. Tennikova, Evgenia G. Vlakh and Anna A. Kulminskaya

http://dx.doi.org/10.1002/biot.201400417

Rapid Communication

Site-specific conjugation of an antibody-binding protein catalyzed by horseradish peroxidase creates a multivalent protein conjugate with high affinity to IgG

Kosuke Minamihata, Masahiro Goto, and Noriho Kamiya

http://dx.doi.org/10.1002/biot.201400512