Disease-Resistant Transgenic Animals

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Glossary

Disease resistance/ susceptibility
The interplay of the genetically determined ability of an individual to prevent the reproduction of a pathogen or to reduce pathogen growth, host-pathogen interactions, and changing environmental conditions/ factors decides on resistance/susceptibility.

Gene targeting
Integration of exogenous DNA into the genome of an organism at specific sites as a result of homologous recombination. It can be used to disrupt or delete a gene, to remove or add sequences, as well as to introduce point mutations at a given locus. Gene targeting can be permanent, i.e., ubiquitous with respect to tissue and developmental stage, or conditional, i.e., restricted to a specific time during development/life or limited to a specific tissue.

Genetic engineering
Technological process resulting in a directed alteration of the genotype of a cell or organism. It combines recombinant nucleic acid technologies; in vitro culture technologies for gametes, embryos, tissues, or organisms; methods for the delivery of nucleic acids to the host genomes (gene transfer); and, if needed, reproductive technologies to produce transgenic embryos and transfer them to foster organisms. With respect to inheritance ("transmission") to offspring, germline and somatic gene transfer methods are distinguished.

Knockdown
Downregulation of expression of a specific gene by RNAi-based technologies.

Knockout/knockin
Incorporation of a sequence into a specific site by homologous recombination (gene targeting) that results in disruption of gene function/ altered gene function.

Quantitative trait loci (QTL)
Genetic loci or chromosomal regions that contribute to variability in complex traits, as identified by statistical analysis. The genetic basis of these traits generally involves the effects of multiple genes and gene-environment interactions.

RNA interference (RNAi)
The silencing of gene expression by the introduction of dsRNA that triggers the specific degradation of a homologous target mRNA, often accompanied by an attendant decrease in the production of the encoded protein.

Single nucleotide polymorphism (SNP)
A variation in DNA sequence in which one nucleotide position is substituted for another by either nucleotide exchange, deletion, or insertion. SNPs are the most frequent type of polymorphism in the genome.

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Somatic cell nuclear transfer (SCNT)	The nonsexual generation of nuclear genome-identical offspring ("cloned animals") by reconstitution of an enucleated oocyte with the diploid nucleus of a somatic cell to a zygote, which under appropriate culture conditions leads to reprogramming of the genome, enabling embryonic and fetal development.
Zoonotic infection	The ability of a given pathogen to cross the host species barrier, from its current or long-term evolutionary host to animals and humans, thereby causing disease.

Definition of the Subject and Its Importance

Infectious diseases of livestock are a major risk to global animal health and welfare. In addition, human health is influenced due to the zoonotic potential of some of these infections.

Moreover, livestock diseases significantly impair food production and safety and cause enormous economic losses worldwide.

Transgenic technology was first developed as a research tool for studying gene function in mice in the early 1980s. The technique was extended and applied to other mammals in 1985. An interesting and challenging focus of agricultural transgenesis was the potential to increase disease resistance and/or reduce disease susceptibility by introducing new genes and/or deleting deleterious genes. The laborious improvements to original and recently developed transgene technologies lead to the generation of transgenic farm animals with improved resistance to infectious diseases, demonstrating the proof of principle that genetic engineering may potentially improve animal health and aid infectious disease control in livestock.

Introduction

Phenotype-driven traditional animal breeding and marker-assisted selection based on quantitative trait loci (QTLs) have been successfully used for the genetic improvement of many agricultural production traits such as body weight, carcass composition, or milk yield. However, these genetic selection strategies have not yet resulted in a significant increase in the resistance of farm animals to disease.

Currently, genomic sequences are available for several livestock species [1], and as a by-product of the sequencing, a huge number of single nucleotide polymorphisms (SNPs) were discovered. The large panels of available SNPs were used in genome-wide association (GWA) studies for mapping and identifying genes [2]. GWA studies have already been successful in identifying causal genes and mutations for monogenic traits [3], but not for complex or quantitative traits such as resistance or susceptibility to disease.

Furthermore, traditional strategies in combating devastating infectious diseases of livestock, such as vaccination, antibiotic treatment, or even culling, have, to date, been unsuccessful (Fig. 1). Parasites evolved to resist chemical or vaccine control measures and bacteria developed resistance to many antibiotics. So far, a single infectious viral disease in livestock, rinderpest (cattle plague), could be eliminated through large-scale vaccination.

As an alternative to the traditional approaches, genetic engineering of livestock species may assist in the fight against infectious diseases.

The oldest and probably the most robust technique to produce transgenic farm animals is the injection of DNA sequences into the pronucleus of recently fertilized zygotes [4–6]. Pronuclear microinjection was
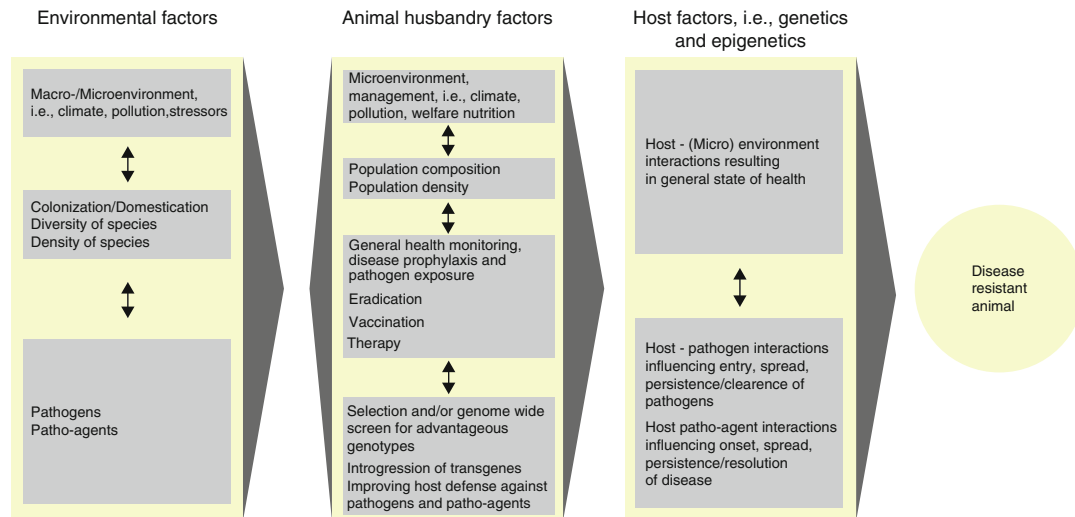
Fig. 1 Horizontal and vertical interrelations influencing the animal's health status, diseased versus nondiseased. The most specific measures to disrupt the pathogen/patho-agent flow toward the animal and/or improve the animal's disease defense mechanisms are highlighted

successfully used to generate the most important livestock species, mainly for production of highly valuable human therapeutics. A more recent method for generating transgenic animals is the nuclear transfer technology, that is, "cloning" [7, 8], which, together with a gene-targeting strategy, allows the generation of specific gene-targeted animals [9, 10]. Recently, lentiviral vector-based strategies have been established which results in highly efficient production of transgenic livestock [11, 12]. This method in combination with the RNAi technology may lead to the generation of disease-resistant transgenic livestock in the near future [13].

In the following section, the authors present an overview of the various transgenic methods used for the genetic enhancement of animal resistance to infectious diseases. Many studies were initially done using transgenic mouse models as this model often provides useful preliminary results prior to initiation of livestock studies.

Disease-Resistant Transgenic Animals

Reducing farm animal susceptibilty to infectious diseases via genetic engineering has been an ambitious goal since the first transgenic livestock was generated more than 20 years ago. Various transgenic strategies for improving animal health are described elsewhere [14–17].

In general, disease-resistant transgenic farm animals can be generated by two approaches: (1) introduction of resistance genes into the genome of the host (gain-of-function strategy) and (2) specific targeting of endogenous or exogenous susceptibility genes (loss-of-function or exchange-of-function strategy).

Improving Animal Health Through Gain-of-Function Gene Transfer

In most cases, susceptibility to pathogens originates from the interplay of numerous genes, meaning susceptibility to pathogens is polygenic in nature. The murine *Mx* gene is one of the few examples of a single genetic (monogenic) locus encoding a disease-resistance trait. Mice and mouse fibroblast cell lines carrying the autosomal dominant *Mx1* allele are resistant to influenza virus infection [18, 19]. The transfer of the *Mx1* gene was able to restore virus resistance in mice lacking the *Mx1* allele [20] and inhibited influenza virus replication in avian cells [21]. However, the introduction of the murine *Mx1* gene into

swine via pronuclear microinjection failed to produce influenza-resistant pigs [22]. The constitutive *Mx1* expression seemed to be detrimental to the pigs, whereas the expression from an inducible promoter was too low to produce detectable levels of *Mx1* protein. In the meantime, *Mx* genes of different farm animals have been identified, but their importance for disease susceptibility is not yet clear [23–25]. However, the ongoing detailed deciphering of the genomes of different farm animals, the improved techniques in generating transgenic animals [26–28], and the new tools for controlling transgene expression levels [29, 30] might allow the idea of generating influenza-resistant livestock by transferring a disease-resistance gene to be addressed once more.

Antimicrobial peptides (AMPs) are an important component of the innate defense of most living organisms, and there is a growing body of evidence to show that their role in defense against microbes is as important to the host as antibodies and innate and adaptive immune cells [31, 32]. AMPs are usually composed of 12–50 amino acids and synthesized by microorganisms as well as multicellular organisms, including plants and animals. They can have broad-spectrum antibacterial, antifungal, antiviral, antiprotozoan, and antisepsis properties. In addition to the wide range of these naturally occurring AMPs, many new ones have also been synthesized [33, 34]. Based on three-dimensional structural studies, the peptides are broadly classified into five major groups, namely, (1) peptides that form alpha-helical structures; (2) peptides that form beta-sheets; (3) peptides rich in cysteine residues; (4) peptides rich in regular amino acids, namely, histatin, arginine, and proline; and (5) peptides composed of rare and modified amino acids [35, 36]. They can induce complete lysis of the organism by disrupting the membrane or by perturbing the membrane lipid bilayer, which allows for leakage of specific cellular components as well as dissipating the electrical potential of the membrane.

In initial engineering studies, the endogenous production of antimicrobial compounds in transgenic animals was shown to enhance disease resistance. Recombinant bovine tracheal antimicrobial peptide (bTAP) isolated from milk from transgenic mice showed antimicrobial activity against *Escherichia coli*, without any deleterious side effects in suckling pups [37]. The antimicrobial activity of the synthetic alpha-helical peptide *Shiva 1a* was confirmed in transgenic mice, challenged with *Brucella abortus* [38]. The expression of the recombinant peptide significantly reduced both the bacterial colonization and the associated pathological changes in the genetically engineered mice.

Mastitis which is caused by bacterial infection of the mammary gland is reported to be the most costly disease in animal agriculture. It seriously affects animal well-being and is the most common reason for antibiotic use in dairy cattle and the most frequent cause of antibiotic residues in milk [39]. The major contagious mastitis pathogen, *Staphylococcus aureus*, is sensitive to lysostaphin, an antibacterial peptide naturally produced by a related bacterium, *Staphylococcus simulans* [40]. Kerr and colleagues showed that mammary gland expression of a bioactive variant of lysostaphin conferred protection against *S. aureus* infection in mice [41]. The staphylolytic activity in the milk of transgenic mice appeared to be fivefold to tenfold less active than bacterially derived lysostaphin but was sufficient to confer substantial resistance to staphylococcal mastitis. Transgene production appeared to have no apparent effect on the physiology of the animal, the integrity of the mammary gland, or the milk it produces. Using nuclear transfer techniques, this approach was successfully extended to cattle, recently [42]. Transgenic dairy cows secreting lysostaphin constitutively in their milk were more resistant to *S. aureus* infections than nontransgenic animals. Lysostaphin concentrations in the milk of transgenic animals remained fairly constant during lactation. The recombinant lysostaphin was approximately 15 % as active as bacterially derived protein. Challenge studies with *S. aureus* clearly demonstrated a direct correlation between the extent of protection against *S. aureus* infection with lysostaphin levels in the milk. Transgenic cows have been previously generated, primarily as bioreactors for large-scale production of pharmaceuticals and nutraceuticals. Thus, lysostaphin-transgenic cattle are the first example for enhancing disease resistance and animal welfare in livestock and may allow substantial reductions in antibiotic use. This in turn will

help to control the spread of antibiotic-resistant bacteria and to reduce bacterial and antibiotic contamination of milk and milk products.

The antibacterial effect of lysostaphin is restricted to *S. aureus* only and transgenic cows are not protected against other mastitis-causing pathogens. The additional expression of secondary antibacterial compounds in the milk might be necessary for further enhancing mastitis resistance.

Human lysozyme (hLZ), a bacteriostatic milk protein that is known to attack the peptidoglycan component of bacterial cell walls, was expressed in the mammary gland of transgenic mice [43] and transgenic dairy goats [44]. Milk from the transgenic animals showed significant bacteriostatic activity and slowed the growth of several bacteria responsible for causing mastitis and the cold-spoilage of milk. The somatic cell count (SCC) is applied as a measure for udder health and milk quality, and a high SCC in milk is directly correlated with mastitis and an impairment of milk quality [45]. Analyzing the SCC in milk samples of transgenic dairy goats revealed a significant lower SCC compared to milk samples from control animals suggesting an improved udder health in the transgenic animals [46]. Lysozyme plays a role in the defense against gastrointestinal pathogens and reduces gastrointestinal illness in breastfed infants [47]. Feeding trials were conducted in pigs to evaluate putative health-promoting functions of hLZ-transgenic milk. Pigs are monogastric animals with a digestive system similar to humans and therefore are commonly used to study human health. Brundige and colleagues demonstrated that the consumption of pasteurized milk from hLZ-transgenic goats improved the gastrointestinal health of young piglets and was beneficial against a gastrointestinal infection with enteropathogenic *E. coli* [48].

A Chinese group enabled synthesis and secretion of bioactive bovine lactoferrin and bovine tracheal antibacterial peptides in goat mammary cells by use of plasmid-mediated gene transfer techniques [49], and the milk samples collected from these animals exhibited bacteriostatic effects against different mastitis-causing pathogens.

The authors summarize that genetic engineering for secretion of a broad range of AMPs in the mammary gland of dairy goats and cows reduces susceptibility to various microbial pathogens and is therefore a realistic approach to combat mastitis. Enhanced mastitis resistance will not only improve animal health and well-being but also reduce bacterial contamination of milk and milk products in addition to reducing the costs incurred during disease prevention and cure.

Transgenic mice, expressing and processing a human enteric alpha-defensin peptide exclusively in specialized epithelia of the small intestinal crypt, were generated and were immune to an oral challenge with virulent *Salmonella typhimurium* [50].

Protegin-1 (PG-1) that is normally expressed in porcine myeloid cells and resides in secretory granules of neutrophils is another potent antimicrobial peptide targeting both gram-negative and gram-positive bacteria [51]. The ectopic expression of PG-1 in transgenic mice conferred enhanced respiratory resistance to an intranasal challenge with *Actinobacillus suis* [52], an opportunistic pathogen that may cause pneumonia, abortion, and fatal septicemia in pigs of all ages [53, 54]. Extending this concept to pigs and other somatic tissues beyond neutrophils will be another step toward the development of disease-resistant livestock.

The overexpression of dominant-negative mutants of viral proteins or pathogen receptors is another potent strategy to enhance animal disease resistance. The major focus has been to block viral attachment and penetration into a host cell by (1) producing viral proteins that block cellular receptors (antireceptor) or (2) altering known host molecular components, such as replacing host receptor genes with a modified version which is able to perform the receptor's physiological function but prevents attachment of the virus [55]. The first successful introduction of pathogen-mediated disease resistance in animals was reported 20 years ago. Transgenic chickens expressing the viral envelope of a recombinant avian leukosis retroviral genome were resistant to the corresponding subgroup of avian leukosis virus due to blockage of the virus receptors by the viral envelope proteins [56]. Using the same strategy, Clements et al. generated transgenic

sheep expressing the maedi-visna virus envelope (E) gene, which is responsible for virus attachment to the host cells [57]. Maedi-visna virus is a prototype of ovine lentiviruses that cause encephalitis, pneumonia, and arthritis in sheep. Transgenic lambs expressing the viral E glycoprotein in monocytes/macrophages, the target cells for virus replication, were healthy, and neither deleterious effects nor clinical abnormalities from the transgene were observed. However, up to date, challenge studies to determine the susceptibility of these animals to ovine lentiviruses have not been reported.

Transgenic mice expressing a soluble form of porcine nectin-1, the cellular receptor for α−herpesviruses, were generated. These mice displayed high resistance to pseudorabies virus (PRV) infections [58]. In pigs, PRV causes lethal encephalitis, acute respiratory syndrome, abortion and infertility, and latent infections [59]. Analysis of transgenic mouse lines, ubiquitously expressing different soluble forms of the cellular receptor for the viral glycoprotein D, revealed that the transgene encoding the soluble form of the entire ectodomain of porcine nectin-1 fused to the human IgG1 conferred highest resistance to intranasal and intraperitonal PRV infections without any side effects [60]. Surprisingly, the expression of a fusion protein consisting of the first Ig-like domain of nectin-1 and the Fc portion of porcine IgG1 not only resulted in reduced virus resistance but also caused microphthalmia and the lack of vitreous bodies [61, 62]. Before implementing this promising approach to the generation of α−herpesviruses-resistant swine, further investigations examining the interactions of different soluble forms of nectin-1, endogenous nectins, and viral glycoprotein D and analysis of the influence of Fc domains of different species are required.

An alternative transgenic approach to protect livestock against infectious diseases is the expression of genes directing the synthesis of defined antibodies which target specific pathogens and thus induce immediate immunity without prior exposure to that pathogen.

Initial studies to express gene constructs encoding monoclonal antibodies in transgenic livestock were conducted nearly 20 years ago [63, 64]. However, the recombinant antibodies expressed in transgenic rabbits, sheep, and pigs showed aberrant sizes and only low antigen-binding affinity. Nevertheless, following this idea, transgenic mice expressing coronavirus-neutralizing antibodies in the mammary gland were generated [65, 66]. High antibody expression titers throughout the lactation period provided complete protection against the enteric infection of newborns with transmissible gastroenteritis virus (TGEV), a pathogen which produces high mortality in suckling piglets, and also against a murine hepatitis virus (MHV)-induced encephalitis. Following this strategy, manipulating the lactogenic immunity in farm animals could improve the protection of suckling newborns through colostrium-delivered antibodies [67].

Enhancing Disease Resistance by Targeting Endogenous Susceptibility Genes

Transmissible spongiform encephalopathies (TSE) are fatal neurodegenerative disorders of the central nervous system which are termed scrapie in goat and sheep and bovine spongiform encephalopathy (BSE) in cattle. According to current knowledge, the causative agent of the brain pathology in diseased animals is the prion. Prion diseases are characterized by the accumulation of the abnormally folded and protease-resistant isoform (PrP^{Sc}) of the cellular prion protein (PrP^C) of the host [68, 69]. The generation of prion-free livestock resistant to TSE has been an ambitious goal since the BSE epidemic in cattle in the UK and the appearance of a new and highly lethal variant of Creutzfeldt-Jakob disease (vCJD) in humans. Early studies in mice revealed that reduction or loss of PrP^C expression did not affect normal development of the mice, but conferred protection against scrapie disease after inoculation with PrP^{Sc} prions [70–73]. With the development of nuclear transfer cloning techniques using genetically modified embryonic or somatic cell donors [7–10], the possible "knockout" of the prion gene in transgenic sheep, goats, and cattle has opened new perspectives for the generation of disease-resistant livestock. A decade ago, Denning and colleagues generated the first PrP^C-targeted lambs. However, none of the cloned sheep survived more than 12 days [74]. Analyses of the targeted fetuses and lambs revealed defects that have been described in other

nuclear transfer experiments with nontransfected cells, and therefore, the authors expected that the early death of the lambs was not a consequence of the PrP^C disruption per se, but was probably due to the nuclear transfer procedures and/or the prolonged culture and drug selection of the primary fibroblasts used for nuclear transfer.

The functional disruption of the caprine PrP^C gene in cloned goats was first described by Yu et al. [75] and resulted in two goats lacking the prion protein [76]. The scientists confirmed the complete PrP^C ablation at mRNA and protein levels, and at 2 months age, the PrP^C-null goats were healthy and showed no developmental or behavioral defects. The scientific community is awaiting the final proof of the concept – scrapie resistance of PrP^C-deficient goats after infection with PrP^{Sc} prions.

Richt and colleagues described the generation of the first PrP^C-deficient cattle [77]. They used a sequential gene-targeting strategy which was demonstrated for the first time by the group of Kuroiwa et al. [78]. Male Holstein primary fibroblasts were transfected with two knockout vectors to sequentially disrupt the two alleles of the PrP^C gene. PrP^C-deficient fetal cell lines were established at 40–75 days of gestation and recloned for the generation of calves. The impact of PrP^C deficiency on calf development, on the immune system, on growth, and on the general health of the cattle for at least 20 months was analyzed in detail, and no negative influence of PrP^C ablation on animal health and well-being was detected. Importantly, brain homogenates from 10-month-old PrP^C-deficient cattle prevented PrP^{Sc} propagation in vitro, whereas in brain homogenates from wild-type cattle PrP^{Sc} proliferated. The researchers concluded that the presence of the endogenous bovine PrP^C is essential for PrP^{Sc} propagation and that there are no other host-derived cellular factors that can support the in vitro PrP^{Sc} propagation in the absence of the endogenous bovine PrP^C. In vivo tests of resistance to prion propagation in PrP^C-deficient cattle are under way but still will require some years to complete. Analyses of several PrP^C-targeted mouse lines indicated that the loss of the normal cellular function of PrP^C may adversely affect the animals. For example, PrP^C-deficient mice developed ataxia and cerebellar neurodegeneration [79, 80], slight alterations in sleep-wake circadian rhythm [81], and altered synaptic functions [82]. To date, none of the above-described alterations in PrP^C-null mice could be observed in PrP^C-deficient cattle and goats, respectively, but further investigations on aged transgenic animals will be necessary to exclude these altered phenotypes.

Small interfering RNAs (siRNAs) can silence/shut down specific targeted genes by interfering with the RNA transcripts they produce [83, 84]. For a transient gene "knockdown," synthetic siRNAs can be directly transfected into cells or early embryos. However, for stable gene expression and germline transmission, the siRNA sequences are incorporated into gene constructs which express short hairpin (sh)RNAs that are processed to siRNAs within the cell. Through stably integrated shRNA expression vectors, additional genetic information is introduced into an organism (gain-of-functions strategy), which then produces a "knockdown" phenotype that is functionally similar to a "knockout" (loss of function). Thus, RNAi transgenics is an interesting alternative to the homologous gene-targeting strategies which are traditionally used for the generation of "knockout" livestock.

One of the most interesting susceptibility genes in livestock is the PrP^C gene, and in a preliminary in vitro experiment, it was demonstrated that siRNA suppression of the PrP^C gene abrogates the PrP^C synthesis and inhibits the formation of PrP^{SC} protein in chronically scrapie-infected murine neuroblastoma cells [85]. Shortly after, Golding and colleagues combined this RNAi-based technique with lentiviral transgenesis for targeting the PrP^C gene in an adult goat fibroblast cell line, which was then used for somatic cell nuclear transfer to produce a cloned goat fetus [13]. Protein analyses of brain tissues demonstrated that PrP^C expression was reduced >90 % in the cloned transgenic fetus when compared with a control. In a further experiment, they injected the recombinant lentivirus directly into the perivitelline space of bovine ova. Development of more than 30 % of injected ova to blastocysts and expression of the shRNA targeting the PrP^C gene in more than 70 % provides strong evidence that this

RNAi approach may be useful in creating genetically engineered farm animals with natural resistance to prion diseases.

In two further approaches, lentiviral-mediated delivery of shRNA expression vectors into the brain of scrapie-infected mice resulted in a clear reduction of the PrP^C protein level and a prolonged survival of infected mice [86, 87], inferring that RNAi technology may also be used for therapeutic applications.

Enhancing Disease Resistance by Targeting Exogenous Susceptibility/Viral Genes

Another application of the RNAi technology is the silencing of exogenous viral genes through the introduction of specific dsRNA molecules into cells, where they are targeted to essential genes or directly to the viral genome, thus inhibiting viral replication [88, 89]. Currently, the use of RNAi-based strategies for generation of viral disease-resistant livestock focuses on three pathogens: food and mouth disease virus (FMDV), bovine viral diarrhea virus (BVDV), and influenza A viruses.

FMDV is an extremely contagious pathogen that affects cattle, swine, and other livestock worldwide [90]. FMD is difficult to control by vaccination and impossible to eliminate by conservative natural breeding. Initial studies tested specific FMDV-siRNAs for their ability to inhibit virus replication in BHK-21 cells [91]. Transfection of BHK-21 cells with a mixture of siRNAs targeting highly conserved sequences of the 3B region and the 3D polymerase gene in all FMDV serotypes resulted in nearly 100 % suppression of virus growth.

In another approach, siRNAs were designed to specifically target the viral VP1 gene, which plays a key role in virus attachment. This resulted in a nearly 90 % reduction in FMDV VP1 expression and conferred resistance to FMDV challenge in cultured cells which are susceptible to this virus [92]. Encouragingly, pretreatment with siRNAs before infection made suckling mice significantly less susceptible to FMDV, and expression of siRNAs directed against the viral nonstructural protein 2B clearly inhibited virus replication in infected porcine cells [93].

Another RNAi target of agricultural interest is the bovine viral diarrhea virus (BVDV), an ubiquitously occurring pathogen that affects cattle herds worldwide resulting in respiratory disorders and increased susceptibility to other pathogens [94]. Lambeth and his group demonstrated that BVDV replication in bovine cells can be efficiently suppressed by RNAi [95]. They transfected shRNA expression vectors and siRNAs targeting the 5€^{2} nontranslated region (NTR) and the region encoding the C protein of the viral genome into MDBK cells. After challenging with BVDV, they detected reduced virus titers by both siRNA- and shRNA-mediated RNAi.

Farm animals, in particular swine and poultry, serve as key links between the natural reservoir of influenza A viruses and epidemics and pandemics in human populations. Due to repeated reassortment or mixing of RNA segments between influenza viruses from different species, virulent strains emerge periodically and often lead to devastating human catastrophes [96]. However, the emergence of the RNAi technology has opened many new options for preventing influenza virus infections in animals.

In initial studies, a set of siRNAs specific for conserved regions of the influenza virus genome could potently inhibit virus production in MDCK cells and embryonated chicken eggs [97]. In subsequent approaches, this strategy was extended to an established animal model of influenza infections by two independent groups. Tompkins and colleagues used siRNAs for targeting highly conserved regions of the viral nucleoprotein (NP) and acidic polymerase (PA). After administration of influenza virus-specific siRNAs via hydrodynamic i.v. injection [98], BALB/c mice were infected intranasally with influenza A/H1N1. Virus titer in lung homogenates was significantly reduced in siRNAs-treated mice when compared to control mice 48 h p.i [99]. In addition, they demonstrated that influenza-specific siRNA treatment can protect mice from otherwise lethal virus challenges.

Ge and coworkers administered influenza virus-specific siRNAs intravenously along with lentiviral shRNA expression vectors into C57BL/6 mice. They demonstrated that siRNAs as well as shRNAs can

reduce influenza virus production in the lung when given either before or after virus infection and that the simultaneous use of two or more siRNAs specific for different virus genes resulted in a more severe reduction of virus titers [100].

A promising approach for the generation of influenza-resistant livestock was published by Wise and colleagues [101]. They used shRNA expression vectors, targeting the viral NP and PA gene for lentiviral-mediated generation of transgenic mice. Expression of the siRNAs was confirmed by an RNAse protection assay, and thus far, stable transmission of the transgene was observed up to the third generation. Currently, transgenic mice are mated to generate homozygous lines for delivering the final proof for influenza virus resistance in vivo.

Recently the generation of transgenic chicken expressing a shRNA molecule able to inhibit influenza virus polymerase activity [102] was reported. Although the transgenic chicken did not exhibit a higher resistance to high challenge doses of H5N1, a highly pathogenic avian influenza virus, they showed strongly reduced transmission of the infection to transgenic and even nontransgenic birds housed in direct contact with them, demonstrating that this strategy may be used to prevent transmission and propagation of an infection at the flock level.

The first transgenic mouse model was described which applied RNAi technology to generate a significant antiviral activity without detectable side effects [103]. Geneviève Jolivet and Louis-Marie Houdebine generated lines of transgenic mice stably expressing shRNA or miRNA targeting the 5′part and 3′UTR of the immediate early gene (IE180 mRNA) of pseudorabies virus (PRV). Upon challenge with PRV, the survival rate of the transgenic animals was higher than in control mice, and the first death occurred later in the transgenics compared to wild types. There was a clear correlation between siRNA expression levels and resistance to viral infection, and it was shown that the antiviral effects of the siRNA were not mediated by nonspecific innate immune mechanisms. As PRV infects many mammalian species including farm animals and is still a serious health and economic problem, this strategy may be a step forward to resolve/minimize these problems.

Future Directions

The past decade was dominated by large-scale and high-throughput nucleic acid analyses allowing comparative genome sequencing and expression profiling projects. The comprehensive and ongoing analysis of the huge data sets led to the need for an updated definition of the term "gene" and the introduction of the term "epigenetics." Taking into account that Mendel's and Morgan's elements of heredity include multifunctional protein coding, structural, regulatory, and RNAs of unknown functions and gene regulation is more complex than previously assumed, the "gene" is suggested to be "a union of genomic sequences encoding a coherent set of potentially overlapping functional products" [104] and "epigenetics" is defined to describe "stably inheritable phenotypes resulting from changes in a chromosome without alterations of the DNA-sequence" [105]. The future challenge of the postgenomic era is subsumed as integrative, quantitative, and/or systems biology. "Systems biology is the comprehensive and quantitative analysis of the interactions between all of the components of biological systems over time" [106]. "Systems biology involves an iterative cycle, in which emerging biological problems drive the development of new technologies and computational tools" [106]. The further understanding of disease mechanisms also depends on these emerging disciplines.

The ongoing genome sequencing programs for various animal species and the increasing densities of SNP arrays will lead to the discovery of new QTLs underlying economically important traits such as disease resistance and susceptibility. In addition, complete genome sequences of many disease-causing pathogens are becoming available. Hence, genome data on host intrinsic factors and host-pathogen

interactions causing disease can be used to increase the health of individuals or populations. Conventional breeding and genomic selection will increasingly benefit from the natural variations identified among the populations. This can be supplemented with gene transfer technologies allowing a more targeted approach toward desired animal breeding without the limitation of species barriers.

The future of transgene technologies is dependent on the simplification of the gene delivery systems along with targeted manipulation of animal genomes. The former aim is achieved by using lentiviral vectors which are highly efficient for domesticated animals including poultry [107, 108] and pets [109]. Gene targeting in species other than mice is limited as embryonic stem (ES) cells of farm or pet animals are unavailable and gene targeting via homologous recombination of embryonic and somatic cells and subsequent nuclear transfer is highly inefficient. However, the advent of the RNAi technology offers new possibilities for specific gene targeting in animal species and will have a huge impact on transgenesis in the near future. Furthermore, the zinc-finger nuclease (ZNF) technology has shown to be an attractive alternative to ES cell targeting and nuclear transfer technology [110] and was already applied successfully for targeted gene disruption in rats [111].

For further reading concerning the use of ZFN technology in farm animals, we refer to Kues and Niemann [112]. In addition, site-directed mutagenesis of genomes can be achieved by TALENs (transcription activator-like effector nucleases) which were originally identified in plant pathogens and recently were successfully used to generate knockout rats [113]. These site-specific nucleases may complement/enlarge the well-established ZFN technology for efficient gene targeting in livestock [114].

Last but not least, the cross-species generation of pluripotent/embryonic cell lines has gained new impetus through the induced pluripotent stem cell (iPS) technology, i.e., the reprogramming of somatic cells making them capable of embryogenesis (reviewed in [115]) and the recent isolation of authentic embryonic stem cells from rat blastocysts by novel culture conditions [116, 117]. In the future, animal transgenetics and animal disease resistance will be important in basic research and in the understanding of disease mechanisms. Bridging the gap between model and man by generating transgenic animals is fundamental to the development of novel therapeutics and disease prevention strategies.

Increased availability of genomic information of livestock species along with more sophisticated transgenic tools offers the potential to generate animal models to combat livestock diseases to a larger extent than ever before. However, animal geneticists/scientists must consider several important aspects. (1) The dissemination of the trait of interest such as disease resistance, introduced by a transgene, will neither be simple nor fast; therefore, cost-benefit calculations will probably decide on implementation of transgenic animals. For example, transgenic BSE-resistant cattle [77] will probably never gain importance in agriculture where culling is considered to meet demands with respect to cost efficiency and biosafety. However, BSE-resistant cattle may be engineered for the production of pharmaceuticals and therefore will have an enormous impact on providing safer drugs. (2) There is general public opposition to the use of transgenic livestock. However, if animals were resistant to zoonotic diseases, therefore resulting in reduced frequency of pandemics and epidemics such as those caused by influenza virus, attitudes of human societies might change [118]. In this context, recently, a trypanosome lytic factor (TLF) from baboons that protected mice both from animal- and human-infective *Trypanosoma* subspecies was identified and suggested to be transferred to livestock [119]. Animal trypanosomiasis is one of the major parasitic diseases of livestock flocks, and livestock are the major reservoir for human-pathogenic trypanosomes. (3) Scientists and society should clearly keep in mind that pathogens readily change their antigenic determinants and create novel subtypes to escape the "resistant" host's immune system. Attempts to introduce resistance traits into animal populations either by conventional breeding or transgenesis should be subjected to thorough cost-/detriment-benefit analyses.

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