Part I
Application and Perspectives

Genetically Engineered Food. Methods and Detection. 2nd Edition
Edited by Knut J. Heller

ISBN: 3-527-31393-1

1
Transgenic Modification of Production Traits in Farm Animals

Gottfried Brem and Mathias Müller

1.1
Introduction

"Genetic engineering" is the umbrella term for procedures that result in a directed alteration in the genotype of an organism. The combined use of molecular genetics, DNA recombination and reproductive biology enables the generation of transgenic animals. For animals the term "transgenic" originally referred to the stable introduction of new genetic material into the germ line [1, 2]. This definition of transgenic animals has to be extended for two reasons. First, further developments in the genetic engineering of animals enable not only additive gene transfer (gain of function) but also deletive gene transfer (knock-out, loss of function), replacement gene transfer (knock-in, exchange of function), and spatial–temporal gene transfer (conditional knock-out) [3, 4]. Second, in addition to germ line integration of transgenes, somatic gene-transfer approaches result in (mostly transient) gene expression with the longest duration being a life span and no transmission of the transgenes to the progeny. Although somatic gene-transfer experiments in farm animals have been performed for production purposes [5, 6] this technology in animal production is more beneficial for the development of DNA-based vaccines [7]. Here we will mainly concentrate the discussion on germ line transgenic animals. The production of transgenic farm animals was first reported in the mid-1980s [8, 9]. The main progress in exploiting this technology has been made in the establishment of animal models for human diseases, production of heterologous proteins in animals (gene farming), and the production of organs for xenotransplantation [10–12]. In addition to these biomedical approaches research has focused on improvement of the efficiency and quality of animal production by transgenic means (this review and other work [13, 14]).

Genetically Engineered Food. Methods and Detection. 2nd Edition
Edited by Knut J. Heller

ISBN: 3-527-31393-1

1.2
The Creation of Transgenic Animals

The main routes to transgenesis in mammals include:

1. microinjection of DNA into the pronucleus of a fertilized oocyte (zygote);
2. integration of a (retro)viral vector into an oocyte or an early embryo;
3. incorporation of genetically manipulated pluripotent stem cells into an early embryo; or
4. transfer of genetically altered nuclei into enucleated oocytes.

For alternative gene transfer methods we refer to another review [15]. Sperm-mediated gene transfer, at least for pigs, has been proven to result in high-efficiency transgenesis ([16] and Refs. therein), although the method is not as broadly established as the methods described below.

1.2.1
Pronuclear DNA Microinjection

Microinjection of foreign DNA into pronuclei of zygotes is the classic method of gene transfer into farm animals. Since the first reports [8, 9] this technique, nearly unchanged, has accounted for most transgenic farm animals [15, 17]. DNA microinjection results in random integration of the foreign DNA into the host genome and is therefore not suitable for targeted modification of genomes. Although microinjections are performed at the one-cell-stage approximately 20–30% of the founder animals are mosaic and, therefore, may not transmit the integrated gene construct to their progeny [18]. Random integration of the gene constructs may cause alteration of one or more gene loci. Insertional mutagenesis is recessive and mostly characterized by a recombination event in the kilobase range at the transgene integration site. In mice approximately 5–15% are affected by this recessive mutation [19]. Except for studies in transgenic rabbits [20, 21], little has been published on analysis of homozygous transgenic farm animals. This is mainly because of the long generation intervals. There is, however, no reason to doubt the mutagenesis frequencies estimated for mice in other transgenic mammals generated by the same technology. Random integration of the gene constructs may also result in varying, aberrant, or abolished transgene expression, because of effects of the adjacent chromatin overcoming the transgene's regulatory sequences. One possible means of avoiding these integration site-dependent effects is the transfer of large DNA constructs, which can form functionally independent chromatin domains [22]. The first successful example for this strategy in livestock was the generation of transgenic rabbits harboring yeast artificial chromosomes (YACs) [23]. An alternative means of protecting transgenes from chromosomal position effects is the use of boundary elements (e.g. insulators, locus control regions, matrix attachment regions) in the gene constructs to achieve copy number-dependent and promoter-dependent and position-independent expression of transgenes [24]. Although success with this strategy has been reported, the effects of the elements were not always as expected.

Gene transfer efficiency (transgenic newborns and/or microinjected zygotes) is usually rather low, especially for large animals. One transgenic animal can be expected after microinjection of 40, 100, 90–110, and 1600 zygotes in mice, pigs, small ruminants, and cattle, respectively [25]. Differences in efficiency emphasize fundamental differences in the reproductive biology of species. Hence a high level of technical skills and experience in embryo collection and embryo transfer are critical for efficient transgenic production. This applies for all gene-transfer programs, however.

As mentioned above, the procedures for generating large mammals by DNA microinjection have remained basically unchanged and little improvement in DNA-transfer efficiency has been achieved. After DNA microinjection, embryos are transferred to synchronized foster mothers. Great progress has been made in the field of embryo transfer. For all farm animal species embryo transfer has been facilitated by the development of endoscopy-guided minimally invasive techniques, reducing stress to the foster mothers and maximizing embryo survival and pregnancy rates [26–29]. Methods are currently being developed with the objective of improving the *in-vitro* production (IVP) of embryos [30, 31]. Endoscopic embryo transfer and IVP is also advantageous in the gene transfer methods discussed next.

1.2.2 Retroviral Vectors

The first germ-line transgenic mice were produced by retroviral infection of early embryos [32]. Retroviruses can be considered natural vehicles for gene delivery to mammalian cells. Endogenous retroviruses (ERVs) are a subset of retro-elements which represent up to 10% of the mammalian genome [33]. The capability of ERVs to reintegrate into the genome through reverse transcription mechanisms results in continuous insertion of new ERVs into the host genome. Until recently, retroviral vectors were not considered for farm-animal transgenesis, because of biosafety concerns and the dependence of most retroviruses on dividing cells for integration into the host genome. Retroviral gene transfer therefore often results in genetic mosaics when developing embryos are infected. With the development of replication-defective retroviral vectors mainly for gene-therapy purposes, a powerful tool for gene transfer in mammalian cells has been established [34]. To avoid mosaicism Chan et al. [35] inoculated bovine oocytes in the final stage of maturation with retroviral vectors. They achieved a remarkably high rate of transgenesis and, as expected, no mosaic transgenic cattle. A similar approach resulted in the generation of transgenic piglets [36]. One major limitation of retroviral vectors is their limited cloning capacity (<10 kb). Gene constructs, however, grow larger and larger to omit variegated transgene expression (see above). A second problem with many retroviral vector-mediated transgenesis relates to transcriptional shutdown of the transgenes [37]. Lentiviral vectors are a new generation of retroviral vectors which, in contrast with standard oncoretrovirus-based vectors, are reported to escape transcriptional silencing. In addition, lentiviruses are able to infect both dividing and

nondividing cells (reviews are available elsewhere [38, 39]). Germ-line transmission and expression of transgenes delivered by lentiviral vectors to one-cell mouse embryos has been reported [40]. Subsequently lentiviral gene transfer has been successfully used in swine [38, 39] and cattle [41]. This technique of transgenesis is more efficient and cost-effective, and technically less demanding, than pronuclear injection. The obstacle of the limited size of the constructs to be transferred remains, however. In the same way as for gene transfer by pronuclear injection, retrovirus-mediated gene transfer can only be used for additive gene transfer and also bears the danger of insertional mutagenesis. Analysis of transgene expression in lentiviral transgenic pigs revealed that, in contrast with early reports, some transgenics showed epigenetic silencing [42].

1.2.3
Pluripotent Stem-cell Technology

Pluripotent stem cells are capable of developing into many cell types, including germ cells, on fusion with pro-implantation embryos (morulae, blastococysts). Pluripotent stem cells can be maintained in tissue culture and genetically manipulated and selected *in vitro* before reconstitution of the embryo. With mice, handling of pluripotent cells has become a routine method for targeted modification of the genome by homologous recombination, i.e. deletive or replacement gene transfer [43]. Numerous efforts to establish pluripotent stem cells in species other than mice have so far failed. Possible reasons for this are discussed elsewhere (Refs [44, 45] and references cited therein). Since nuclear transfer using transgenic donor cells (see below) became an attractive alternative tool for targeted gene transfer, efforts to establish germ-line-competent stem cells from farm animals have been reduced.

1.2.4
Nuclear Transfer Using Transgenic Cells

Nuclear transfer technology – also known as cloning – comprises transfer of a donor nucleus (karyoplast) into the cytoplasm of an enucleated zygote or oocyte (cytoplast). Initial nuclear transfer experiments in farm animals used early embryonic stages as nuclear donors [46]. In breakthrough experiments with sheep it was demonstrated that *in-vitro*-cultured differentiated fetal cells and even cells derived from adult tissues could serve as nuclear donor for the reconstitution of enucleated oocytes [47, 48]. Cloning by nuclear transfer has subsequently been achieved in cattle [49–51], goats [52], pigs [53–55], rabbits [56], mules [57], horses [58], cats [59], dogs [60], and some wildlife species (a review is available elsewhere [61]). For farm animal transgenesis a novel tool has become available in that cultured cells can be genetically modified by conventional transfection methods before their use for nuclear transfer. The first reports on this novel gene transfer technique were the generation of transgenic sheep and cattle by nuclear transfer using transfected and selected fetal fibroblasts [62, 63]. Transgenesis by nuclear transfer of geneti-

cally modified cells has several advantages over the other additive gene transfer techniques:

1. mosaicism is avoided and germ-line transmission is guaranteed, because all cells of the cloned animal contain the transgene;
2. insertional mutagenesis and chromosomal positioning effects can be avoided, because integration and, eventually, transgene expression can be monitored *in vitro*; and
3. the use of male or female cell lines determines the gender of the transgenic animal.

Most importantly, gene transfer by nuclear transfer provides a means of gene targeting in farm-animal species [64, 65]. Both the targeted disruption of genes by homologous recombination (deletive gene transfer, knock-out) in sheep and pigs [66–68] and the targeted integration of a gene of interest into a given locus (replacement gene transfer, knock-in) [69] have been reported. The sequential targeting of both alleles of two genes has been achieved in cattle [70].

Despite these impressive reports and the intriguing advantages of the nuclear transfer technique for generation of transgenic farm animals, widespread use is not an easy task because:

1. the primary fibroblasts currently used for gene transfer have limited capacity to divide;
2. homologous recombination is less frequent in somatic cells than in pluripotent stem cells; and
3. cloning by nuclear transfer has a low yield which is still diminished when nuclear donor cells are previously cultured [61].

There is also an ongoing debate about whether it is possible to overcome abnormalities observed in cloned animal [71, 72]. The abnormalities are not restricted to transgenic cloned animals, suggesting they originate from the nuclear transfer procedure or the *in vitro* culture conditions. Although healthy clones have been reported [73], improvement of the technology and further investigation of the effects of cloning are required [74].

1.3 Gene Transfer in Poultry

Depending on the developmental stage a variety of strategies are used for generation of transgenic birds, including DNA microinjection of fertilized ova, retroviral infection of blastodermal cells, and genetic manipulation of primordial germ cells (PGCs) or embryonic stem (ES) cells. In the same way as in mammals, the first method developed to transfer genes into birds was microinjection of DNA into the germinal disk of fertilized ova [75]. Although successful germ line transmission has been reported [76], the method is labor-intensive, ineffective, and frequently results in mosaicism. Retroviral vectors can introduce transgenes into the genome with low but acceptable efficiency. The first transgenic birds were produced using replication-competent vectors and thus could not be used for broad appli-

cation [77]. The development of replication-defective vectors led to wide use of this technique for production of transgenic birds and stable transgene expression [78, 79]. As an alternative, chimeras bearing transfected pluripotent cells originating from the blastoderm, from PGCs, or from ES cells have been reported (reviews are available elsewhere [80–82]).

1.4 Gene Transfer in Fish

The techniques for gene transfer into fish have focused on direct transfer of DNA into gametes or fertilized eggs and include DNA microinjection, electroporation, retroviral vector infection, and biolistic methods (Ref. [83] and references cited therein). Stem-cell-based technology is not available for farmed fish. The making of transgenic fish is different from gene transfer in mammals or birds because:

1. fish usually undergo external fertilization and no culture or transfer of eggs into recipient females is required;
2. the eggs of many fish have a tough chorion requiring special methods for delivering the gene constructs; and
3. DNA delivery, including by microinjection, is usually into the cytoplasm.

Probably because of the cytoplasmic nature of DNA delivery, many founder transgenic fish are mosaic. Germ line mosaicism seems also to occur because frequencies of transgene transmission to F1 are clearly less than at Mendelian ratios. Transmission of the transgenes to later progeny occurs at Mendelian frequencies, indicating stable integration of the transgenes. A variety of inducible and targeted transgene strategies developed for mammals are now available to be tested and explored in fish (a review is available elsewhere [84]).

1.5 Transgenes – Gene Constructs

The exogenous DNA integrated into the host genome usually is referred to as the gene construct or transgene. The different transgenic sequences used for the different gene-delivery methods and gene-transfer programs are summarized in Table 1.1.

For additive gene transfer experiments the gene construct encompasses the elements controlling gene expression (5′ promoter region, 3′ control regions) and the sequences (cDNA, genomic DNA) encoding the transgene product. For DNA microinjection or sperm-mediated gene transfer the prokaryotic cloning vector sequences are removed from the gene construct. Prokaryotic sequences – especially their CpG dinucleotide base pairs – may undergo methylation or heterochromatin formation in animal cells, which leads to transgene silencing. It is becoming increasingly clear that silenced transgenes have been recognized as foreign elements by host cellular mechanisms, as have retroviral and transposon sequences [85, 86].

Tab. 1.1 Characteristics of the transgenes used for different gene-delivery methods and gene-transfer programs in farm animals.

Functional consequence of gene transfer	Methods of gene transfer	Sequences not related to the transgene per se	Composition and origin of the transgene	Example (see below)
Gain of function	Pronuclear DNA micro-injection; sperm-mediated; physical and/or chemical methods	–	• Species-specific sequences • cross-species sequences • new combination of promoter and coding sequences (species-specific or cross-species)	• Additional copies of casein alleles • humanized milk • mammary gland-specific expression of antibodies
	Artificial nonmammalian chromosomes	PAC-, BAC-, YAC-vector elements	See above	See above
	Artificial mammalian chromosomes	Chromosomal elements	See above	See above
	Retroviral vector	Viral sequences	See above	See above
	Cloning by transfer of genetically modified nuclei	Selectable marker genes*	See above	See above
Loss of function	Cloning by transfer of genetically modified nuclei	Selectable marker genes*	Deleted or non-sense-mutated species-specific sequences	Generation of PrPc gene-deficient ruminants
Exchange of function	Cloning by transfer of genetically modified nuclei	Selectable marker genes*	• Introduction of novel allelic variants • exchange of coding sequences in a transcription unit	• Targeted alteration of milk protein genes • replacement of genes
Reduction of function by RNAi	Pronuclear DNA micro-injection; sperm-mediated; physical and/or chemical methods; retroviral vectors	RNAi expression construct	RNAi expression construct gene(s)	Specific "knock-down" of target

* Unwanted sequences may be removed *in vitro* by site-specific recombinases

As mentioned above, one obvious way of avoiding transgene silencing or chromosomal positioning effects would be the use of large gene constructs and the abandoning of viral vectors. An increasing number of transgenic animals therefore carry gene constructs based on phage (PAC), bacterial (BAC), or yeast (YAC) artificial chromosomes [22]. For expression and replication, these large transgenes depend on integration into the host genome. In contrast, mammalian artificial chromosomes (MACs) provide independent transcription and replication units. Work originating mainly from human gene therapy programs has resulted in the development of human artificial chromosomes based on episomal viral vectors or engineered minimal chromosomal elements [87, 88]. MACs were recently used to generate "transchromosomic" cattle; this was achieved by introducing human artificial chromosomes *in vitro* into bovine cells which were subsequently used for cloning by nucleus transfer [89, 90].

Alternatively, gene transfer *in vitro* then reconstitution of embryos by nuclear transfer or stem-cell technology enables targeting of transcription units in the host genome or *in-vitro* analysis of the chromosomal integration site. These gene-transfer techniques, however, require methods for the identification of the genetically modified cells. Identification of transgenic cells is mostly based on (drug) selectable markers, e.g. antibiotic-resistance genes, added to the gene constructs. In plants the presence of marker genes in the genetically modified organism is a topic of concern about biosafety [91]. Analogous a genetically modified farm animal going on the market should be free from such genes. This can be achieved by use of site-specific recombinases to remove undesirable sequences after successful identification of the transgenic cells [92, 93]. Additional methods for site-directed genome modification are reviewed elsewhere [94, 95]. It must, however, be mentioned that these additional genetic engineering steps have not yet been conducted in farm animals.

RNA interference (RNAi) is the process in which dsRNA leads to gene silencing, by either inducing the sequence-specific degradation of complementary mRNA or inhibiting translation. RNAi has been very successfully applied as gene-silencing technology in both plants and invertebrates, but many practical obstacles need to be overcome before it becomes a versatile tool in mammalian cells. Greater specificity and efficiency of RNAi in mammals is being achieved by improving the design and selection of small interfering RNAs (siRNAs), by increasing the efficacy of their delivery to cells and organisms, and by engineering their conditional expression (reviews are available elsewhere [4, 96, 97]).

The power of this technology in transgenic animals has been demonstrated by production of mice constitutively expressing RNAi directed against a given transcript which show a similar phenotype as mice with homologous targeted disruption of the locus [98].

Retroviral vector-mediated gene transfer methods have the advantage of the transgene being actively delivered to the cells and integrated into the host genome. The disadvantage of the system is the above mentioned transcriptional shutdown and heterochromatin formation of the transgenes, because of the presence of the viral sequences.

1.6
Transgenic Animals with Agricultural Traits

A key element of the enhanced production of domesticated species is the development of genetically superior breeding stocks tailored to their maintenance conditions and to the marketplace. Characteristics that are generally desirable in all species include improvement of growth rates, feed conversion efficiency, disease resistance, and a capacity to utilize low-cost or nonanimal protein diets. Attempts to improve productivity traits in farm animals by transgenesis can be divided into products designed for consumers' consumption *per se* and traits not affecting the food chain in the first place. The first topic includes stimulation of growth rates, food conversion, and alteration of carcass and milk composition. The second aims at improvement of fiber products, enhanced disease resistance, and introduction of novel biochemical pathways. Although the transgene product in this field is not meant for food, the meat or milk of the genetically modified animal could be considered for consumption (reviews are available elsewhere [11, 14, 99]).

At the outset it should be noted that progress on manipulation of agricultural animal traits is far slower than originally envisaged in the early days of transgene technology. First, this is because the most economically important traits are controlled by multiple genes, which are still largely unknown and hence not amendable to manipulation. Even after identification of all genes contributing to a complex trait, genetic engineering of this trait would require multiple gene transfers. Second, the low efficiency of gene transfer in farm animals (see above) makes research on transgenesis quite costly. Third, the ability to regulate expression of transgenes is still far from adequate (see above). Finally, public acceptance of genetically modified organisms in the food chain – at least in Europe – has not yet been achieved.

Compared with mammals and fish gene-transfer experiments in chickens are still limited. The objectives of gene transfer in poultry are basically identically with those for other farm animals (reviews are available elsewhere [100, 101]).

1.7
Improved Growth Rate, Carcass Composition, and Feed Efficiency

1.7.1
Transgenic Mammalian Farm Animals

Among the genetically determined factors regulating growth rate and feed conversion the genes encoding polypeptides of the growth-hormone cascade are of particular interest. The positively acting growth hormone-releasing hormone (GHRH, somatoliberin) and its antagonist somatotropin release-inhibiting factor (SRIF, somatostatin) control the production of growth hormone (GH, somatotropic hormone (STH), somatotropin). The action of GH is very dependent on the metabolic state of the organism – low blood glucose levels result in catabolic effects (lipolysis)

and a positive energy balance causes anabolic effects which are mainly governed by insulin-like growth factor 1 (IGF-1, somatomedin C). The original workers on farmanimal transgenesis was impressed by the results of Palmiter et al. [102] which indicated that mice expressing excess GH grew much faster and bigger than nontransgenic control mice. Several GH transgenic pigs and sheep have been produced with human, bovine, ovine, porcine, or rat GH, under the control of several promoters (a review is available elsewhere [14]). GH-transgenic pigs expressing the gene constructs at high levels were found to have faster growth rates and increased feed efficiency. The most dramatic effect of elevated GH levels in pigs was reduction of carcass fat as transgenic pigs approached market weight [13, 103]. It was found, however, that constitutive and/or high level expression of GH in pigs had a variety of pathological side-effects [104, 105]. Transgenic ruminants (cattle, sheep, goats) carrying growth-promoting genes have been also generated but no positive effects on growth performance or carcass composition were reported (a review is available elsewhere [14]). Attempts to affect carcass composition in respect of beneficial dietary properties for human consumption are, nevertheless, of high priority for the meat-production industry (Section 1.10, below and Ref. [106]).

It was recognized that tight regulation of transgene expression would be required to avoid deleterious effects from continuous exposure of mammals to elevated GH. So far, most attempts to use dietary inducible promoters have failed. Two studies reported the production of growth-promoting transgenic pig lines. A metal ion-inducible promoter linked to the porcine GH gene was introduced into pigs and many transgenic founder animals were produced. Transgenic pigs were tested for metal-induced transgene expression and animals with high basal levels of transgene expression or plasma GH levels outside the range of non-transgenics were excluded from the study. By following this strategy negative side-effects could be avoided [107]. Because of the random integration of the transgenes as a result of DNA microinjection and the lack of shielding sequences, however, the transgenic lines had a high level of variegated gene expression. In a second study the expression of IGF-1 was directed to muscle by using a skeletal muscle-specific expression cassette. By avoiding the systemic effects of GH increased carcass leanness was achieved with no detrimental side-effects [108]. Interestingly, in the context of the use of growth hormone cascade transgenes, somatic gene-transfer might satisfy demands for improved growth performance. A somatic gene-transfer procedure employing a singular intramuscular injection and electroporation of muscle-specific expression vectors encoding a protease-resistant GHRH resulted in elevated GH and IGF-1 levels which, in turn, augmented long-term growth without pathological side-effects [6].

An alternative means of altering the growth performance involves the differentiation process of muscle cells themselves. For example, the chicken c-ski proto-oncogene was found to induce myogenic differentiation. This muscle differentiation gene was introduced into cattle [109]. As observed with the growth hormone cascade genes, no effects or mainly deleterious effects of the transgene expression were reported, however. Myostatin, also known as growth and differentiation factor 8, negatively regulates skeletal muscle development. Deletion or

non-sense mutations in the myostatin gene are responsible for double muscling in cattle (a review is available elsewhere [110]). Myostatin-deficient mice, generated by gene targeting, produced twice the muscle mass with reduced carcass fatness as wild type mice, mirroring the phenotype of myostatin mutant cattle [111]. In an additive gene transfer experiment it was shown that expression of a dominant-negative myostatin transgene also led to increased muscle development, growth performance, and carcass weight [112]. By use of a two-step procedure involving insertional gene targeting and recombinase-mediated cassette exchange in ES cells, transgenic mice were generated which express a dominant-negative myostatin propeptide under control of a muscle and in a Y-chromosome-specific manner. Transgenic males were characterized by a 5–20% increase in skeletal muscle mass. This experiment demonstrates the feasibility of a more efficient cattle production system combining superior beef production for bulls and dairy output for cows [113]. The myostatin gene is therefore an attractive candidate for both gene targeting and gain of function experiments in farm animals [110].

1.7.2 Transgenic Fish

Aquaculture is still in its infancy compared with the farming of mammals or poultry. Growth rates of the many fish species used are naturally slow but are currently being enhanced by traditional methods of domestication and selection. Programs for growth-promoting gene-transfer into fish usually use GH-based gene constructs [83]. Because of the lack of available piscine sequences, the first experiments were conducted with mammalian GH gene constructs. The effects on growth performance were, however, either not detectable or very small. Gene transfer using fish GH sequences driven by nonpiscine promoters has resulted in growth stimulatory effects in transgenic carp, catfish, and tilapia; weight increases were approximately twice those of controls. These experiments provided the first consistent data demonstrating that growth acceleration in fish can be achieved by transgenesis [83]. Subsequent use of all-piscine gene constructs produced fish with up to fortyfold elevated circulating GH levels and five- to elevenfold increased weight after one year of growth [114, 115]. Pleiotropic effects in the GH-transgenic fish included altered body composition (50% reduced fat levels), unpredictable variations in food consumption and conversion, and some pathological side-effects. Comparative gene-transfer programs demonstrated that GH-transgenes dramatically enhanced the growth of wild but not domesticated fish [116]. Thus in domesticated and selected farm animal species the capacity for further growth enhancement by GH may be restricted by limitations in other physiological pathways. In mammals this is reflected by dramatic growth stimulation in GH-transgenic mice but not in domestic livestock that have undergone many centuries of genetic selection (see above). Genetically engineered fish with enhanced phenotypic traits have yet to be implemented in commercial applications. In addition to the technical issues described, this is partly because of the difficulties in reliably predicting the ecological risk of transgenic fish should they escape into the wild. The ecological

consequences of the phenotypic differences between transgenic and wild-type fish, as determined in the laboratory, can be uncertain, because of genotype-by-environment effects. These biosafety issues are reviewed elsewhere [117].

Salmonids are fish of high economic value which are unable to survive in waters characterized by ice and subzero temperatures. Antifreeze proteins (AFP) are produced by several fish that inhabit extremely cold waters. One possible way of solving the problem of overwintering salmon in sea cages in the northern hemisphere is the transfer of antifreeze protein genes. The AFP-transgenic salmons produced so far express the transgene at levels insufficient to confer freeze resistance (a review is available elsewhere [83]).

1.8 Alteration of the Composition of Milk

Potential changes in milk composition or in the primary structures of milk proteins, and their presumed beneficial effects on the nutritional, physiochemical, and technological properties of milk and milk products have already been reviewed extensively [14, 118].

It should be noted that most transgenic strategies are at the stage of being tested by use of mouse models and gene-transfer technology for improvement of milk quality is far from commercial application. Attempts to improve the processing properties of milk include changing the casein content and the introduction of modified milk proteins. To enhance milk composition and milk processing efficiency by increasing the casein concentration in milk, cloning by transgenic female nucleus transfer was used to generate transgenic cows carrying additional copies of bovine beta-casein and kappa-casein genes. Analysis of the hormonally induced milk revealed substantial expression and secretion of the transgene-derived caseins into milk. These results show it is feasible to substantially alter a major component of milk in high-producing dairy cows by a transgenic approach and thus to improve the functional properties of dairy milk [119].

Introduction of human milk protein genes or replacement of bovine genes by human genes may play an important role in the production of surrogates for human milk. Bovine and human milk differ substantially and, therefore, cows' milk is not an ideal source of food for babies. Bovine milk can be humanized by increasing the whey protein content, e.g. by expressing the antimicrobial proteins lactoferrin and lysozyme [120, 121]. Large-scale production of human lactoferrin in the milk of transgenic cows has recently been reported [122]. The structure of the transgenic protein was reported to match that of human origin [123]. Beta-lactoglobulin is the major heat-labile whey protein of ruminant milk and does not occur in human milk. Although beta-lactoglobulin is not the only bovine milk protein with allergenic properties it is widely assumed that milk depleted of this protein would be a better source of humanized milk. In principle, the technology for gene knock-out in ruminants is now available. Depletion of particular proteins from the milk has been performed in mice for beta-casein [124] and alpha-lactalbumin

[125, 126]. Whereas beta-casein was found to be a non-essential component of the milk protein system, knock-out of alpha-lactalbumin resulted in disruption of lactation and lactose synthesis. Because the biological function of beta-lactoglobulin and its contribution to bovine milk physiology are not known, the side-effects of a knock-out approach cannot be predicted. RNAi technology – once established in farm animals – will be useful for determining the biology of these proteins and potentially down-regulating their expression.

Tab. 1.2 Proposed modifications of milk composition.

Target gene	Gene transfer	Transgene effect	Overall effect
alpha- and beta-Caseins	Gain of function	Improved cheese-making properties, increased Ca^{2+} content	Improved technical processing
kappa-Casein	Gain of function	Improved heat stability, reduced micelle size, reduced coagulation	Improved technical processing
Novel phosphorylation and proteolytic sites in caseins	Exchange of function	Increased Ca^{2+} content, improved cheese ripening	Improved technical processing
beta-Lactoblobulin	Loss of function	Increased temperature stability, improved digestibility, reduced allergenicity	Improved technical processing and/or humanized milk
Human lysozyme	Gain of function	Antimicrobial, increased cheese yield	Improved technical processing and/or humanized milk
Human lactoferrin	Gain of function	Antimicrobial	Humanized milk
alpha-Lactalbumin	Reduction of function	Reduced lactose	Improved nutritional value
Lactase	Gain of function	Reduced lactose	Improved nutritional value
Acetyl CoA carboxylase	Reduction of function	Reduced fat content	Improved nutritional value
Immunoglobulin genes	Gain of function	Passive oral immunization	Nutraceutical
Antigen genes	Gain of function	Active oral immunization	Nutraceutical
Phe-free alpha-lacta bumin	Exchange of function	Source of amino acids for patients suffering from phenylketonuria	Nutraceutical
Stearoyl-CoA desaturase	Gain of function	Fatty acid pattern of benefit to cardiovascular health	Nutraceutical

Lactose is the major sugar present in milk and is synthesized by the lactose synthase complex comprising a galactosyltransferase and alpha-lactalbumin. Most of the adult population suffers from intestinal disorders as a consequence of lactose maldigestion that results from physiological downregulation, at weaning, of the intestinal lactose-hydrolyzing enzyme. Low-lactose milk *in vivo* has been generated by partial inhibition of the alpha-lactalbumin gene by an RNA-antisense approach [127] and by the mammary gland-specific expression of an intestinal lactase [128].

Milk seems to be an ideal vehicle for development of nutraceuticals, i.e. dietary supplements and functional and medical food [129]. Examples of the use of milk as a nutraceutical are listed in Table 1.2.

The enzyme stearoyl-CoA desaturase converts specific medium-chain and long-chain saturated fatty acids into their monounsaturated forms. Transgenic goats expressing this enzyme in the mammary gland produce milk in which the fat has a less saturated and more monounsaturated fatty acid profile at some stages of lactation. Milk containing higher proportions of monounsaturated fatty acids and conjugated linoleic acid may benefit human cardiovascular health [130].

Bovine alpha-lactablumin gene transgenic pigs have been generated in an attempt to increase milk production and lactose content for the benefit of suckling offspring. Enhanced lactation performance and, consequently, enhanced litter growth performance were observed (a review is available elsewhere [131]).

1.9 Improved Animal Health

In contrast with selection for production performance, attempts to select for improved disease resistance by conventional breeding programs have not been successful. Reduction of the susceptibility of livestock to disease will be of benefit to animal welfare and will also be of economic importance. Improved health status in animal production results in improved production and reproduction performance. Both somatic and germ-line gene transfer can be used. To introduce new disease-resistance traits to farm animals, germ-line transmission is required. Strategies for enhancing disease resistance by transgenesis have been extensively reviewed elsewhere [14, 132, 133]. Somatic gene transfer mainly focuses on DNA vaccines (genetic immunization, see above). Integration, stable germ-line transmission, and, occasionally, expression of gene constructs designed to confer disease resistance have been demonstrated. The final proof of successful generation of resistant farm animals, i.e. challenge with an infectious pathogen, has not, however, been reported until recently. In a breakthrough experiment, genetically engineered cows were shown to have enhanced resistance to mastitis [134]; reviews are available elsewhere [99, 135].

1.9.1 Additive Gene Transfer of Resistance Genes

The term "intracellular immunization" was originally used to denote the overexpression in the host of an aberrant form (dominant-negative mutant) of a viral protein that can interfere severely with replication of the wild type virus. This definition has since been extended to all approaches based on intracellular expression of transgene products which inhibit the replication of pathogens in host organisms (Ref. [133] and references cited therein). Initial studies with farm animals included the "classical" approach of overexpression of a viral protein in transgenic sheep [136], transgenic rabbits expressing antisense constructs complementary to adenovirus RNA [137], and the transfer of the specific disease resistance gene Mx1 of mice into swine [138]. The mouse Mx1 gene is one of the few examples of a single genetic locus encoding a disease resistance trait. Mice carrying the autosomal dominant Mx1 allele are resistant to influenza viruses and transfer of the Mx1 gene into susceptible mice that lacked the Mx1 allele was sufficient to restore virus resistance [139]. Swine are susceptible to influenza and provide a substantial reservoir for swine influenza viruses. Different gene constructs containing the mouse Mx1 cDNA controlled by two constitutive promoters and the inducible murine Mx1 promoter were transferred into swine. Constitutive Mx1 expression was found to be detrimental to the organism and the inducible Mx1 construct responded to stimuli by RNA synthesis, although at levels insufficient to produce detectable amounts of Mx1 protein. This again emphasizes the importance of tight transgene regulation to a positive outcome from gene-transfer experiments [138].

"Congenital immunization" is defined as transgenic expression and germ-line transmission of a gene encoding an immunoglobulin specific for a pathogen and therefore providing congenital immunity without prior exposure to that pathogen. The approach was tested in farm animals by expressing the gene constructs encoding mouse monoclonal antibodies in transgenic rabbits, pigs, and sheep [140, 141]. Both experiments resulted in transgene expression but revealed also some unexpected findings, e.g. aberrant sizes of the transgenic antibody or little antigen-binding capacity. Following this idea, preformed antibodies against a virus causing a neonatal disease were expressed in the mammary gland of mice. Transferred to farm animals this could improve the protection of suckling offspring by use of colostral-delivered antibodies [142]. It remains to be investigated, however, whether the efforts required to optimize the concept of "congenital immunization" are justified by its benefits in terms of increasing disease resistance in a particular species. When following this route one has must also remember that a given infectious pathogen will be readily able to escape the transgenic animal's immunity by changing its antigenic determinants. Transfer of antibody-encoding genes into farm animals is, in general, of great importance for production of therapeutic antibodies for human medicine (Refs [90, 143] and references cited therein).

"Extracellular immunization" refers to transgene products with extracellular antipathogenic function. This strategy includes the systemic or local expression of immunomodulatory cytokines and pathogen defense molecules of the innate

immune system [132]. Peptide-based antimicrobial defense is an evolutionarily ancient mechanism of host response found in a wide range of animals from insects to mammals. The small lytic peptides interact with lipid bilayer membranes to cause osmotic disruption and cell death. Bacterial, protozoan, fungal, and damaged eukaryotic cells are most susceptible to disruption [144]. Experiments with transgenic mice and fish have revealed the power of overexpressing antimicrobial peptides in generating enhanced resistance of the animals against invading microbes [145–147]. Lysostaphin is a potent bacterial peptidoglycan hydrolase with specific bactericidal activity against *Staphylococcus aureus*, the major contagious mastitis pathogen. Mammary gland-specific expression of lyostaphin could therefore enable production of mastitis-resistant cows and reduce the major economic burden of the dairy industry. Proof of principle of mammary gland-expressed lysostaphin conferring protection against stapylococcal infection has been obtained for mice [148]. By use of gene transfer via cloning by nuclear transfer this concept was successfully extended to cows [134]. Transgenic mice expressing a soluble form of a protein known as herpesvirus entry mediator were generated. The transgenic mice had nearly complete resistance to challenge with the alpha-herpesvirus, suggesting the potential of the approach for generating pseudorabies-resistant livestock [149].

1.9.2 Gene Targeting of Susceptibility Genes

Another potential means of improving disease resistance is the removal of disease susceptibility genes by homologous recombination to create null alleles or to replace a disease allele by a resistance allele [14]. This concept is discussed for generation of cattle and sheep resistant to spongiform encephalopathies caused by infectious prion particles. Mice devoid of the endogenous prion protein PrPc cannot be infected with the infectious particles and the loss of function mice now have gross abnormalities [150]. Targeted inactivation of the gene encoding PrPc in cattle and sheep might therefore create BSE or scrapie-resistant animals. Successful targeting of the PrP locus in sheep and bovine fetal fibroblasts for use of nuclear transfer has been reported, and living PrP-deficient sheep and cattle are to be obtained [67, 70]. The physiological role of the endogenous prion protein and the genetic components of susceptibility to the disease are still largely unknown. (More information is available elsewhere [151, 152]). Future experiments will show whether fundamental differences between mice and ruminants will prevent the generation of such loss of function animals.

An attractive alternative to gene targeting by homologous recombination is provided by RNAi technology which, in future, should enable modulation or downregulation of the genes involved in disease processes (see the discussion above and Ref. [153]).

1.10
Improved Biochemical Pathways

Transgenesis enables the transfer of genetic information across species barriers. Combined with functional promoter elements, nonmammalian genes can be expressed in farm animals to modify intermediary metabolism. To address the problem of environmental pollution with manure in pig production, transgenic pigs were produced which express the *Escherichia coli* phytase gene in the salivary gland. Consequently, the transgenic pigs can digest the phosphorus in phytate, the most abundant source of phosphorus in the pig diet, and so fecal phosphorus excretion by transgenic pigs is substantially reduced [154]. "Phosphorus-friendly" pigs contributing to solution of a common form of environmental problem might also help overcome public skepticism toward transgenic livestock (see below and Ref. [155]).

The introduction of new biochemical pathways to increase the availability of specific nutrients that are currently rate-limiting in animal production is a challenging task. Research programs on transferring prokaryotic genes to ruminants to introduce cysteine, threonine, and lysine biosynthesis or a functional glyoxylate cycle (for conversion of the major rumen metabolite acetate to glucose) have so far failed [156].

The first example of a plant gene expressed in a complex mammalian system was provided by Saeki et al. [157]. The work of the group is part of an international effort to improve pig meat quality (carcass composition, see also above). A fatty acids desaturase gene from spinach, functionally expressed in pigs, led to an significant increase of polyunsaturated fatty acids in pork. This success may pave the way toward production of healthier porcine meat and to diversification of the range of products available from livestock [106, 157].

1.11
Improved Wool Production

Improvement of wool production by transgenesis can be achieved by generating an abundant supply of the cysteine required for keratin synthesis. Keratins are the major structural proteins of wool fibers and cysteine is the rate-limiting amino acid in wool production. Dietary addition of cysteine does not increase wool production because of digestive degradation of the compound. When bacterial cysteine biosynthesis genes were transferred into sheep, however, improved wool growth was not observed, because transgene regulation was not adequate to integrate the novel pathway into the existing biochemical homeostasis [158].

The second attempt at improving wool quality was directed at modification of the protein composition of the wool fiber. The gene encoding the wool intermediate filament keratin was overexpressed in transgenic sheep and alteration of the fiber ultrastructure was observed. The changes did not have a positive effect on the processing quality of the wool [159]. Further investigations are in progress, using

transgenes encoding keratins rich in the amino acids which have a positive effect on wool quality [160].

The third approach used to improve wool production is the targeted overexpression of growth factors (EGF, IGF-1) in wool follicles or skin [161, 162]. So far, none of the approaches has resulted in a novel transgenic breed with improved wool production traits.

1.12 Transgenic Farm Animals, Biosafety Issues, Animal Welfare, and Ethics

The biosafety of transgenics concerns the consumer and affects the environment and the transgenic animals. Evaluation of the food safety of transgenic animal products (novel food, nutraceuticals) is handled by national and international authorities (a review is available elsewhere [129]). Theoretically, meat or milk from transgenic farm animals generated for nonfood purposes could also be consumed. For example, the transgene in the "phosphorus-friendly" pig is expressed exclusively in the salivary gland; the meat, therefore, contains only the transgene DNA not the transgene product. For consumers' safety, the risk of toxicity and of transfer of the ingested transgenes must be assessed. DNA is an essential part of nutrition and is not usually toxic upon uptake through the digestive tract. Large alimentary DNA fragments seem to survive gastrointestinal passage, enter the blood stream, and can be found in the nuclei of a variety of types of cell [163]. It is obvious, however, that this does not lead to vertical gene transfer and there is no reason why a transgene should integrate into a consumer's genome preferentially compared with nonrecombinant DNA. Nevertheless, especially with regard to the relevance and frequency of transfer of selectable DNA sequences from genetically modified plants in the gut of mammals to bacteria the debate is still ongoing (Ref. [164] and references cited therein).

The biorisks resulting from transgenesis in farm animals depend on the animal species, the method of gene transfer, the nature of the transgene, and the fate of the transgenics. Genetically modified organisms are not allowed to multiply uncontrolled in the environment. Large farm animals have little opportunity to escape and no chance of crossing with wild animals. More concern is warranted over possible escape of transgenic growth-enhanced fish from cage culture with access to free waters [165] and subsequent spread of the transgene into the natural population [166]. The fish in such cages must therefore be rendered sterile or they must be kept in artificial containment facilities.

Physiochemical gene-transfer methods and cloning by nuclear transfer do not have intrinsic biorisks, because the DNA is stably integrated into the host genome. Viral vectors are generally liable to the risk of recombination with wildtype viruses that in turn might create the means to spread the transgene.

The major biorisks arising from transgenes are to the animals themselves (see below). All transferred DNA fragments should be characterized by sequencing. This may be not always be possible, however, especially for large gene constructs, for example artificial chromosomes. Transgenic animals designed for human consumption will be devoid of marker genes and other sequences not required for transgene function. Methods for the removal of such sequences are available (see above).

A transgenic founder animal has, *a priori*, unknown biological properties and must be tested for stable integration and transmission of the transgene. The F1 generation can be tested for stable and promoter-specific, i.e. nonectopic expression of the transgene. Homozygous animals are produced to prove the freedom of insertional mutagenesis. It is obvious that before use of the transgenic animal for production purposes wanted and possible unwanted transgene effects are thoroughly checked by laboratory and veterinarian methods. Programs for the systematic assessment of risks associated with transgenic farm animal welfare and breeding have been developed and must be applied rigorously [167].

Environmental issues and animal welfare are the most serious public concerns about animal biotechnology. Scientific uncertainty often hinders good risk assessment of animal biotechnology. Ethical assessments must openly address these uncertainties, with the precautionary principle providing a good criterion for responsible policies. A practical method for ethical assessment of animal biotechnology has been proposed [168].

1.13 Conclusion

Gene-transfer technology enables the direct introduction of novel traits into farm animals. The biological performance of the transgene in the animal can be measured in a few generations. Breeding success can be achieved in a shorter time compared with classical breeding programs. Gene transfer can, however, be only performed in a limited number of animals. When a positive biological outcome of the artificially added or altered allele in the founder animal's offspring is established, the novel trait has to be spread in the production population by conventional strategies. So far no genetically engineered food from farm animals has been made available commercially. This is because of the above mentioned difficulties in the generation of transgenic farm animals, the frequent failure to transfer the proof of principle success in basic research models to farm animals, and the lack of public acceptance of novel food. The reasons for this lack of acceptance, and the ethics of transgenics are discussed elsewhere [168, 169].

Farm animal transgenics is undoubtedly important for biomedicine. Even if public perception moved in favor of transgenic food and if optimized gene-transfer technology increased efficiency in the generation and exploitation of transgenics, most livestock-derived products would still be for niche markets only, mainly because of limitations in the dissemination of the transgene (introgression).

Farm animal transgenics, farm animal reproductive biotechnology, and farm animal genomics have developed in parallel [170–173]. These three important aspects of the technology will develop further in the years to come and the tools, taken together, will eventually lead to genetically modified farm animals that meet the demands of productivity and biosafety.

References

1 Gordon, J.W., F.H. Ruddle, *Science* **1981**, *214*, 1244-1246.
2 Gordon, J.W., G.A. Scangos, D.J. Plotkin, J.A. Barbosa, F.H. Ruddle, *Proc. Natl. Acad. Sci.U S A* **1980**, *77*, 7380-7384.
3 Clark, J., B. Whitelaw, *Nat. Rev. Genet.* **2003**, *4*, 825-833.
4 Ristevski, S., *Mol. Biotechnol.* **2005**, *29*, 153-164.
5 Düchler, M., M. Pengg, S. Schüller, F. Pfneisl, C. Bugingo, G. Brem, E. Wagner, K. Schellande, M. Müller, *J. Gene Med.* **2002**, *4*, 282-291.
6 Draghia-Akli, R., M.L. Fiorotto, L.A. Hill, P.B. Malone, D.R. Deaver, R.J. Schwartz, *Nat. Biotechnol.* **1999**, *17*, 1179-1183.
7 Dunham, S.P., *Res. Vet. Sci.* **2002**, *73*, 9-16.
8 Hammer, R.E., V.G. Pursel, C.E. Rexroad Jr., R.J. Wall, R.D. Palmiter, R.L. Brinster, *Nature* **1985**, *315*, 680-683.
9 Brem, G., B. Brenig, H.M. Goodman, R.C. Selden, F. Graf, B. Kruff, K. Springman, J. Hondele, J. Meyer, E.-L. Winnacker, H. Kräußlich, *Reprod. Dom. Anim.* **1985**, *20*, 251-252.
10 Keefer, C.L., *Anim. Reprod. Sci.* **2004**, *82-83*, 5-12.
11 Kues, W.A., H. Niemann, *Trends Biotechnol.* **2004**, *22*, 286-294.
12 Houdebine, L.M., *Reprod. Domest. Anim.* **2005**, *40*, 269-281.
13 Pursel, V.G., C.A. Pinkert, K.F. Miller, D.J. Bolt, R.G. Campbell, R.D. Palmiter, R.L. Brinster, R.E. Hammer, *Science* **1989**, *244*, 1281-1288.
14 Wheeler, M.B., E.M. Walters, S.G. Clark, *Anim. Reprod. Sci.* **2003**, *79*, 265-289.
15 Wall, R.J., *Theriogenology* **2002**, *57*, 189-201.
16 Webster, N.L., M. Forni, M.L. Bacci, R. Giovannoni, R. Razzini, P. Fantinati, A. Zannoni, L. Fusetti, L. Dalpra, M.R. Bianco, M. Papa, E. Seren, M.S. Sandrin, I.F. Mc Kenzie, M. Lavitrano, *Mol. Reprod. Dev.* **2005**, *72*, 68-76.
17 Houdebine, L.M., *Reprod. Nutr. Dev.* **2005**, *45*, 363-376.
18 Brem, G., M. Müller, *Large transgenic animals*, in *Animals with novel genes*, N. Maclean, Editor. 1994, Cambridge University Press: Cambridge, UK. p. 179-244.
19 Rijkers, T., A. Peetz, U. Rüther, *Transgenic Res.* **1994**, *3*, 203-215.
20 Zinovieva, N., C. Lassnig, D. Schams, U. Besenfelder, E. Wolf, S. Müller, L. Frenyo, J. Seregi, M. Müller, G. Brem, *Transgenic Res.* **1998**, *7*, 437-447.
21 Aigner, B., U. Besenfelder, J. Seregi, L.V. Frenyo, T. Sahintoth, G. Brem, *Transgenic Res.* **1996**, *5*, 405-411.
22 Giraldo, P., L. Montoliu, *Transgenic Res.* **2001**, *10*, 83-103.
23 Brem, G., U. Besenfelder, B. Aigner, M. Müller, I. Liebl, G. Schütz, L. Montoliu, *Mol. Reprod. Dev.* **1996**, *44*, 56-62.
24 West, A.G., M. Gaszner, G. Felsenfeld, *Genes Dev.* **2002**, *16*, 271-288.
25 Wall, R.J., *Theriogenology* **1996**, *45*, 57-68.
26 Besenfelder, U., G. Brem, *Theriogenology* **1998**, *50*, 739-745.
27 Besenfelder, U., J. Mödl, M. Müller, G. Brem, *Theriogenology* **1997**, *47*, 1051-1060.
28 Besenfelder, U., N. Zinovieva, E. Dietrich, B. Sohnrey, W. Holtz, G. Brem, *Vet. Rec.* **1994**, *135*.
29 Besenfelder, U., G. Brem, *J. Reprod. Fert.* **1993**, *99*, 53-56.
30 Wetscher, F., V. Havlicek, T. Huber, M. Gilles, D. Tesfaye, J. Griese, K. Wimmers, K. Schellander, M. Muller, G. Brem, U. Besenfelder, *Theriogenology* **2005**, *64*, 30-40.
31 Havlicek, V., F. Wetscher, T. Huber, G. Brem, M. Mueller, U. Besenfelder, *J. Vet. Med. A Physiol. Pathol. Clin. Med.* **2005**, *52*, 94-98.

32 Jaenisch, R., *Proc. Natl. Acad. Sci. USA* **1976**, *73*, 1260-1264.
33 Temin, H.M., *Genome* **1989**, *31*, 17-22.
34 Pfeifer, A., I.M. Verma, *Annu. Rev. Genomics Hum. Genet.* **2001**, *2*, 177-211.
35 Chan, A.W., E.J. Homan, L.U. Ballou, J.C. Burns, R.D. Bremel, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 14028-14033.
36 Cabot, R.A., B. Kuhholzer, A.W. Chan, L. Lai, K.W. Park, K.Y. Chong, G. Schatten, C.N. Murphy, L.R. Abeydeera, B.N. Day, R.S. Prather, *Anim. Biotechnol.* **2001**, *12*, 205-214.
37 Pannell, D., J. Ellis, *Rev. Med. Virol.* **2001**, *11*, 205-217.
38 Whitelaw, C.B., *Trends Biotechnol.* **2004**, *22*, 157-159; discussion 159-160.
39 Pfeifer, A., *Transgenic Res.* **2004**, *13*, 513-522.
40 Lois, C., E.J. Hong, S. Pease, E.J. Brown, D. Baltimore, *Science* **2002**, *295*, 868-872.
41 Hofmann, A., V. Zakhartchenko, M. Weppert, H. Sebald, H. Wenigerkind, G. Brem, E. Wolf, A. Pfeifer, *Biol. Reprod.* **2004**, *71*, 405-409.
42 Hofmann, A., B. Kessler, S. Ewerling, A. Kabermann, G. Brem, E. Wolf, A. Pfeifer, *Mol. Ther.* **2006**, *13*, 59-66.
43 Capecchi, M.R., *Nat. Genet.* **2000**, *26*, 159-161.
44 Familari, M., L. Selwood, *Mol. Reprod. Dev.* **2006**, *73*, 123-131.
45 Gjorret, J.O., P. Maddox-Hyttel, *Reprod. Fertil. Dev.* **2005**, *17*, 113-124.
46 Willadsen, S.M., *Nature* **1986**, *320*, 63-65.
47 Wilmut, I., A.E. Schnieke, J. McWhir, A.J. Kind, K.H.S. Campbell, *Nature* **1997**, *385*, 810-813.
48 Campbell, K.H.S., J. McWhir, W.A. Ritchie, I. Wilmut, *Nature* **1996**, *380*, 64-66.
49 Wells, D.N., P.M. Misica, H.R. Tervit, *Biol. Reprod.* **1999**, *60*, 996-1005.
50 Zakhartchenko, V., V. Alberio, M. Stojkovic, K. Prelle, W. Schernthaner, P. Stojkovic, H. Wenigerkind, R. Wanke, M. Düchler, R. Steinborn, M. Müller, G. Brem, E. Wolf, *Mol. Reprod. Dev.* **1999**, *54*, 264-272.
51 Kato, Y., T. Tani, Y. Sotomaru, K. Kurokawa, J. Kato, H. Doguchi, H. Yasue, Y. Tsunoda, *Science* **1998**, *282*, 2095-2098.
52 Baguisi, A., E. Behboodi, D.T. Melican, J.S. Pollock, M.M. Destrempes, C. Cammuso, J.L. Williams, S.D. Nims, C.A. Porter, P. Midura, M.J. Palacios, S.L. Ayres, R.S. Denniston, M.L. Hayes, C.A. Ziomek, H.M. Meade, R.A. Godke, W.G. Gavin, E.W. Overstrom, Y. Echelard, *Nat. Biotechnol.* **1999**, *17*, 456-461.
53 Betthauser, J., E. Forsberg, M. Augenstein, L. Childs, K. Eilertsen, J. Enos, T. Forsythe, P. Golueke, G. Jurgella, R. Koppang, T. Lesmeister, K. Mallon, G. Mell, P. Misica, M. Pace, M. Pfister-Genskow, N. Strelchenko, G. Voelker, S. Watt, S. Thompson, M. Bishop, *Nat. Biotechnol.* **2000**, *18*, 1055-1059.
54 Onishi, A., M. Iwamoto, T. Akita, S. Mikawa, K. Takeda, T. Awata, H. Hanada, A.C. Perry, *Science* **2000**, *289*, 1188-1190.
55 Polejaeva, I.A., S.H. Chen, T.D. Vaught, R.L. Page, J. Mullins, S. Ball, Y. Dai, J. Boone, S. Walker, D.L. Ayares, A. Colman, K.H. Campbell, *Nature* **2000**, *407*, 86-90.
56 Chesné, P., P.G. Adenot, C. Viglietta, M. Baratte, L. Boulanger, J.-P. Renard, *Nat. Biotechnol.* **2002**, *20*, 366-369.
57 Woods, G.L., K.L. White, D.K. Vanderwall, G.P. Li, K.I. Aston, T.D. Bunch, L.N. Meerdo, B.J. Pate, *Science* **2003**, *301*, 1063.
58 Galli, C., I. Lagutina, G. Crotti, S. Colleoni, P. Turini, N. Ponderato, R. Duchi, G. Lazzari, *Nature* **2003**, *424*, 635.
59 Shin, T., D. Kraemer, J. Pryor, L. Liu, J. Rugila, L. Howe, S. Buck, K. Murphy, L. Lyons, M. Westhusin, *Nature* **2002**, *415*, 859.
60 Lee, B.C., M.K. Kim, G. Jang, H.J. Oh, F. Yuda, H.J. Kim, M.H. Shamim, J.J. Kim, S.K. Kang, G. Schatten, W.S. Hwang, *Nature* **2005**, *436*, 641.
61 Campbell, K.H., R. Alberio, I. Choi, P. Fisher, R.D. Kelly, J.H. Lee, W. Maalouf, *Reprod. Domest. Anim.* **2005**, *40*, 256-268.
62 Cibelli, J.B., S.L. Stice, P.J. Golueke, J.J. Kane, J. Jerry, C. Blackwell, F.A. Ponce de Leon, J.M. Robl, *Science* **1998**, *280*, 1256-1258.
63 Schnieke, A.E., A.J. Kind, W.A. Ritchie, K. Mycock, A.R. Scott, M. Ritchie, I. Wilmut, A. Colman, K.H.S. Campbell, *Science* **1997**, *278*, 2130-2133.
64 Wells, K., *Nat. Biotechnol.* **2001**, *19*, 529-530.
65 Piedrahita, J.A., *Theriogenology* **2000**, *53*, 105-116.

66 Dai, Y., T.D. Vaught, J. Boone, S.H. Chen, C.J. Phelps, S. Ball, J.A. Monahan, P.M. Jobst, K.J. McCreath, A.E. Lamborn, J.L. Cowell-Lucero, K.D. Wells, A. Colman, I.A. Polejaeva, D.L. Ayares, *Nat. Biotechnol.* **2002**, *20*, 251-255.

67 Denning, C., S. Burl, A. Ainslie, J. Bracken, A. Dinnyes, J. Fletcher, T. King, M. Ritchie, W.A. Ritchie, M. Rollo, P. de Sousa, A. Travers, I. Wilmut, A.J. Clark, *Nat. Biotechnol.* **2001**, *19*, 559-562.

68 Lai, L., D. Kolber-Simonds, K.W. Park, H.T. Cheong, J.L. Greenstein, G.S. Im, M. Samuel, A. Bonk, A. Rieke, B.N. Day, C.N. Murphy, D.B. Carter, R.J. Hawley, R.S. Prather, *Science* **2002**, *295*, 1089-1092.

69 McCreath, K.J., J. Howcroft, K.H.S. Campbell, A. Colman, A.E. Schnieke, A.J. Kind, *Nature* **2000**, *405*, 1066-1069.

70 Kuroiwa, Y., P. Kasinathan, H. Matsushita, J. Sathiyaselan, E.J. Sullivan, M. Kakitani, K. Tomizuka, I. Ishida, J.M. Robl, *Nat. Genet.* **2004**, *36*, 775-780.

71 Cibelli, J.B., K.H. Campbell, G.E. Seidel, M.D. West, R.P. Lanza, *Nat. Biotechnol.* **2002**, *20*, 13-14.

72 Renard, J.P., Q. Zhou, D. LeBourhis, P. Chavatte-Palmer, I. Hue, Y. Heyman, X. Vignon, *Theriogenology* **2002**, *57*, 203-222.

73 Lanza, R.P., J.B. Cibelli, D. Faber, R.W. Sweeney, B. Henderson, W. Nevala, M.D. West, P.J. Wettstein, *Science* **2001**, *294*, 1893-1894.

74 Wilmut, I., *Nat. Med.* **2002**, *8*, 215-216.

75 Perry, M.M., H.M. Sang, *Transgenic Res.* **1993**, *2*, 125-133.

76 Love, J., C. Gribbin, C. Mather, H. Sang, *Biotechnology* **1994**, *12*, 60-63.

77 Salter, D.W., E.J. Smith, S.H. Hughes, S.E. Wright, A.M. Fadly, R.L. Witter, L.B. Crittenden, *Poult. Sci.* **1986**, *65*, 1445-1458.

78 Iba, H., *Dev. Growth Differ.* **2000**, *42*, 213-218.

79 Harvey, A.J., G. Speksnijder, L.R. Baugh, J.A. Morris, R. Ivarie, *Nat. Biotechnol.* **2002**, *19*, 396-399.

80 Ivarie, R., *Trends Biotechnol.* **2003**, *21*, 14-19.

81 Lillico, S.G., M.J. McGrew, A. Sherman, H.M. Sang, *Drug Discovery Today* **2005**, *10*, 191-196.

82 Stern, C.D., *Dev. Cell* **2005**, *8*, 9-17.

83 Zbikowska, H.M., *Transgenic Res.* **2003**, *12*, 379-389.

84 Rocha, A., S. Ruiz, A. Estepa, J.M. Coll, *Mar. Biotechnol. (NY)* **2004**, *6*, 118-127.

85 Dorer, D.R., *Transgenic Res.* **1997**, *6*, 3-10.

86 Garrick, D., S. Fiering, D.I.K. Martin, E. Whitelaw, *Nat. Genet.* **1998**, *18*, 56-59.

87 Saffery, R., K.H. Choo, *J. Gene Med.* **2002**, *4*, 5-13.

88 Vos, J.M., *Curr. Opin. Mol. Ther.* **1999**, *1*, 204-215.

89 Robl, J.M., P. Kasinathan, E.J. Sullivan, Y. Kuroiwa, K. Tomizuka, I. Ishida, *Theriogenology* **2003**, *59*, 107-113.

90 Kuroiwa, Y., P. Kasinathan, Y.J. Choi, R. Naeem, K. Tomizuka, E.J. Sullivan, J.G. Knott, A. Duteau, R.A. Goldsby, B.A. Osborne, I. Ishida, J.M. Robl, *Nature Biotechnol.* **2002**, *20*, 889-894.

91 Hohn, B., A.A. Levy, H. Puchta, *Curr. Opin. Biotechnol.* **2001**, *12*, 139-143.

92 Baer, A., J. Bode, *Curr. Opin. Biotechnol.* **2001**, *12*, 473-480.

93 Gorman, C., C. Bullock, *Curr. Opin. Biotechnol.* **2000**, *11*, 455-460.

94 Coates, C.J., J.M. Kaminski, J.B. Summers, D.J. Segal, A.D. Miller, A.F. Kolb, *Trends Biotechnol.* **2005**, *23*, 407-419.

95 Kolb, A.F., C.J. Coates, J.M. Kaminski, J.B. Summers, A.D. Miller, D.J. Segal, *Trends Biotechnol.* **2005**, *23*, 399-406.

96 Mittal, V., *Nat. Rev. Genet.* **2004**, *5*, 355-365.

97 Novina, C.D., P.A. Sharp, *Nature* **2004**, *430*, 161-164.

98 Shinagawa, T., S. Ishii, *Genes Dev.* **2003**, *17*, 1340-1345.

99 Maga, E.A., *Trends Biotechnol.* **2005**, *23*, 533-535.

100 Verrinder Gibbins, A.M., *Anim. Biotechnol.* **1998**, *9*, 173-179.

101 Verrinder Gibbins, A.M., *Nat. Biotechnol.* **1998**, *16*, 1013-1014.

102 Palmiter, R.D., R.L. Brinster, R.E. Hammer, M.E. Trumbauer, M.G. Rosenfeld, N.C. Birnberg, R.M. Evans, *Nature* **1982**, *360*, 611-615.

103 Solomon, M.B., V.G. Pursel, E.W. Paroczay, D.J. Bolt, *J. Anim. Sci.* **1994**, *72*, 1242-1246.

104 Pinkert, C.A., E.J. Galbreath, C.W. Yang, L.J. Striker, *Transgenic Res.* **1994**, *3*, 401-405.

105 Ebert, K.M., T.E. Smith, F.C. Buonomo, E.W. Overstrom, M.J. Low, *Anim. Biotechnol.* **1990**, *1*, 145-159.
106 Niemann, H., *Proc. Natl. Acad. Sci. U S A* **2004**, *101*, 7211-7212.
107 Nottle, M.B., H. Nagashima, P.J. Verma, Z.T. Du, C.G. Grupen, S.M. McIlfatrick, R.J. Ashman, M.P. Harding, C. Giannakis, P.L. Wigley, I.G. Lyons, D.T. Harrison, B.G. Luxford, R.G. Campbell, R.J. Crawford, A.J. Robins, *Production and analysis of transgenic pigs containing a metallothionein procine growth hormone gene construct*, in *Transgenic animals in agriculture*, J.D. Murray, et al., Editors. 1999, CAB International: Wallingford UK. p. 145-156.
108 Pursel, V.G., A.D. Mitchell, G. Bee, T.H. Elsasser, J.P. McMurtry, R.J. Wall, M.E. Coleman, R.J. Schwartz, *Anim. Biotechnol.* **2004**, *15*, 33-45.
109 Bowen, R.A., M.L. Reed, A. Schnieke, G.E. Seidel, J.A. Stacey, W.K. Thomas, O. Kajikawa, *Biol. Reprod.* **1994**, *50*, 664-668.
110 Bellinge, R.H., D.A. Liberles, S.P. Iaschi, A. O'Brien P, G.K. Tay, *Anim. Genet.* **2005**, *36*, 1-6.
111 McPherron, A.C., A.M. Lawler, S.J. Lee, *Nature* **1997**, *387*, 83-90.
112 Yang, J., T. Ratovitski, J.P. Brady, M.B. Solomon, K.D. Wells, R.J. Wall, *Mol. Reprod. Dev.* **2001**, *60*, 351-361.
113 Pirottin, D., L. Grobet, A. Adamantidis, F. Farnir, C. Herens, H. Daa Schroder, M. Georges, *Proc. Natl. Acad. Sci. U S A* **2005**, *102*, 6413-6418.
114 Du, S.J., Z. Gong, G.L. Fletcher, M.A. Shears, M.J. King, D.R. Idler, C.L. Hew, *Biotechnology* **1992**, *10*, 176-181.
115 Devlin, R.H., T.Y. Yesaki, C.A. Biagi, E.M. Donaldson, P. Swanson, W.-K. Chan, *Nature* **1994**, *371*, 209-210.
116 Devlin, R.H., C.A. Biagi, T.Y. Yesaki, D.E. Smailus, J.C. Byatt, *Nature* **2001**, *409*, 781-782.
117 Devlin, R.H., L.F. Sundstrom, W.M. Muir, *Trends Biotechnol.* **2005**.
118 Karatzas, C.N., *Nat. Biotechnol.* **2003**, *21*, 138-139.
119 Brophy, B., G. Smolenski, T. Wheeler, D. Wells, P. L'Huillier, G. Laible, *Nat. Biotechnol.* **2003**, *21*, 157-162.
120 Krimpenfort, P., A. Rademakers, W. Eyestone, A. van der Schans, S. van den Broek, P. Kooiman, E. Kootwijk, G. Platenburg, F. Pieper, R. Strijker, H. DeBoer, *Bio/Technology* **1991**, *9*, 844-847.
121 Maga, E.A., G.B. Anderson, M.C. Huang, J.D. Murray, *Transgenic Res.* **1994**, *3*, 36-42.
122 van Berkel, P.H.C., M.M. Welling, M. Geerts, H.A. van Veen, B. Ravensbergen, M. Salaheddine, E.K.J. Pauwels, F. Pieper, J.H. Nuijens, P.H. Nibbering, *Nat. Biotechnol.* **2002**, *20*, 484-487.
123 Thomassen, E.A., H.A. van Veen, P.H. van Berkel, J.H. Nuijens, J.P. Abrahams, *Transgenic Res.* **2005**, *14*, 397-405.
124 Kumar, S., A.R. Clarke, M.L. Hooper, D.S. Horne, A.J.R. Law, J. Leaver, A. Springbett, E. Stevenson, J.P. Simons, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 6138-6142.
125 Stinnakre, M.G., J.L. Vilotte, S. Soulier, J.C. Mercier, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 6544-6548.
126 Stacey, A., A. Schnieke, M. Kerr, A. Scott, C. McKee, I. Cottingham, B. Binas, C. Wilde, A. Colman, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 2835-2839.
127 L'Huillier, P.J., S. Soulier, M.G. Stinnakre, L. Lepourry, S.R. Davis, J.C. Mercier, J.L. Vilotte, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 6698-6703.
128 Jost, B., J.L. Vilotte, I. Duluc, J.L. Rodeau, J.N. Freund, *Nat. Biotechnol.* **1999**, *17*, 160-164.
129 Brower, V., *Nat. Biotechnol.* **1998**, *16*, 728-731.
130 Reh, W.A., E.A. Maga, N.M. Collette, A. Moyer, J.S. Conrad-Brink, S.J. Taylor, E.J. DePeters, S. Oppenheim, J.D. Rowe, R.H. BonDurant, G.B. Anderson, J.D. Murray, *J. Dairy Sci.* **2004**, *87*, 3510-3514.
131 Wheeler, M.B., *J. Anim. Sci.* **2003**, *81 Suppl 3*, 32-37.
132 Muller, M., G. Brem, *Rev. Sci. Tech.* **1998**, *17*, 365-378.
133 Muller, M., G. Brem, *J. Biotechnol.* **1996**, *44*, 233-242.
134 Wall, R.J., A.M. Powell, M.J. Paape, D.E. Kerr, D.D. Bannerman, V.G. Pursel, K.D. Wells, N. Talbot, H.W. Hawk, *Nat. Biotechnol.* **2005**, *23*, 445-451.
135 Donovan, D.M., D.E. Kerr, R.J. Wall, *Transgenic Res.* **2005**, *14*, 563-567.

136 Clements, J.E., L.B. Hu, L. Lindstrom, A. Powell, C. Rexroad, M.C. Zink, *AIDS Res. Hum. Retroviruses* **1996**, *12*, 421-423.

137 Ernst, L.K., V.I. Zakcharchenko, N.M. Suraeva, T.I. Ponomareva, O.I. Miroshnichenko, M.I. Prokof'ev, T.I. Tikchonenko, *Theriogenology* **1990**, *35*, 1257-1271.

138 Muller, M., B. Brenig, E.L. Winnacker, G. Brem, *Gene* **1992**, *121*, 263-270.

139 Arnheiter, H., S. Skuntz, M. Noteborn, S. Chang, E. Meier, *Cell* **1990**, *62*, 51-61.

140 Weidle, U.H., H. Lenz, G. Brem, *Gene* **1991**, *98*, 185-191.

141 Lo, D., V. Pursel, P.J. Linto, E. Sandgren, R. Behringer, C. Rexroad, R.D. Palmiter, R.L. Brinster, *Eur. J. Immunol.* **1991**, *21*, 25-30.

142 Saif, L.J., M.B. Wheeler, *Nat. Biotechnol.* **1998**, *16*, 334-335.

143 Grosse-Hovest, L., S. Muller, R. Minoia, E. Wolf, V. Zakhartchenko, H. Wenigerkind, C. Lassnig, U. Besenfelder, M. Muller, S.D. Lytton, G. Jung, G. Brem, *Proc. Natl. Acad. Sci. U S A* **2004**, *101*, 6858-6863.

144 Zasloff, M., *Nature* **2002**, *415*, 389-395.

145 Yarus, S., J.M. Rosen, A.M. Cole, G. Diamond, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 14118-14121.

146 Reed, W.A., P.H. Elzer, F.M. Enright, J.M. Jaynes, J.D. Morrey, K.L. White, *Transgenic Res.* **1997**, *6*, 337-347.

147 Jia, X., A. Patrzykat, R.H. Devlin, P.A. Ackerman, G.K. Iwama, R.E. Hancock, *Appl. Environ. Microbiol.* **2000**, *66*, 1928-1932.

148 Kerr, D.E., K. Plaut, A.J. Bramley, C.M. Williamson, A.J. Lax, K. Moore, K.D. Wells, R.J. Wall, *Nat. Biotechnol.* **2001**, *19*, 66-70.

149 Ono, E., K. Amagai, S. Taharaguchi, Y. Tomioka, S. Yoshino, Y. Watanabe, P. Cherel, L.M. Houdebine, M. Adam, M. Eloit, M. Inobe, T. Uede, *Proc. Natl. Acad. Sci. U S A* **2004**, *101*, 16150-16155.

150 Bueler, H., A. Aguzzi, A. Sailer, R.A. Greiner, P. Autenried, M. Aguet, C. Weissmann, *Cell* **1993**, *73*, 1339-1347.

151 Weissmann, C., *Cell* **2005**, *122*, 165-168.

152 Weissmann, C., A. Aguzzi, *Annu. Rev. Med.* **2005**, *56*, 321-344.

153 Hunter, C.V., L.S. Tiley, H.M. Sang, *Trends Mol. Med.* **2005**, *11*, 293-298.

154 Golovan, S.P., R.G. Meidinger, A. Ajakaiye, M. Cottrill, M.Z. Wiederkehr, D.J. Barney, C. Plante, J.W. Pollard, M.Z. Fan, M.A. Hayes, J. Laursen, J.P. Hjorth, R.R. Hacker, J.P. Phillips, C.W. Forsberg, *Nat. Biotechnol.* **2001**, *19*, 741-745.

155 Ward, K.A., *Nat. Biotechnol.* **2001**, *19*, 415-416.

156 Ward, K.A., *Trends Biotechnol.* **2000**, *18*, 99-102.

157 Saeki, K., K. Matsumoto, M. Kinoshita, I. Suzuki, Y. Tasaka, K. Kano, Y. Taguchi, K. Mikami, M. Hirabayashi, N. Kashiwazaki, Y. Hosoi, N. Murata, A. Iritani, *Proc. Natl. Acad. Sci. U S A* **2004**, *101*, 6361-6366.

158 Bawden, C.S., A.V. Sivaprasad, P.J. Verma, S.K. Walker, G.E. Rogers, *Transgenic Res.* **1995**, *4*, 87-104.

159 Bawden, C.S., B.C. Powell, S.K. Walker, G.E. Rogers, *Transgenic Res.* **1998**, *7*, 273-287.

160 Bawden, C.S., B.C. Powell, S.K. Walker, G.E. Rogers, *Exp. Dermatol.* **1999**, *8*, 342-343.

161 Damak, S., H.Y. Su, N.P. Jay, D.W. Bullock, *Bio/Technology* **1996**, *14*, 185-188.

162 Powell, B.C., S.K. Walker, C.S. Bawden, A.V. Sivaprasad, G.E. Rogers, *Reprod. Fertil. Dev.* **1994**, *6*, 615-623.

163 Palka-Santini, M., B. Schwarz-Herzke, M. Hosel, D. Renz, S. Auerochs, H. Brondke, W. Doerfler, *Mol. Genet. Genomics* **2003**, *270*, 201-215.

164 Heritage, J., *Nat. Biotechnol.* **2004**, *22*, 170-171.

165 Reichhardt, T., *Nature* **2000**, *406*, 10-12.

166 Muir, W.M., R.D. Howard, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 13853-13856.

167 Van Reenen, C.G., T.H.E. Meuwissen, H. Hopster, K. Oldenbroek, T.A.M. Kruip, H.J. Blokhuis, *J. Anim. Sci.* **2001**, *79*, 1763-1779.

168 Kaiser, M., *Rev. Sci. Tech.* **2005**, *24*, 75-87.

169 Davies, K.G., *Trends Biotechnol.* **2001**, *19*, 424-427.

170 Niemann, H., W. Kues, J.W. Carnwath, *Rev. Sci. Tech.* **2005**, *24*, 285-298.

171 Bulfield, G., *Trends Biotechnol.* **2000**, *18*, 10-13.

172 Fadiel, A., I. Anidi, K.D. Eichenbaum, *Nucleic Acids Res.* **2005**, *33*, 6308-6318.

173 Basrur, P.K., W.A. King, *Rev. Sci. Tech.* **2005**, *24*, 31-49.