

APPLICATION OF RABBITS IN BIOMEDICAL RESEARCH: A REVIEW

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ABSTRACT: The first transgenic rabbits were obtained two decades ago by pronuclear microinjection. Several characteristics of rabbit made it the first and classical model for the study of lipoproteins and atherosclerosis. Rabbit models include normal cholesterol-fed rabbits, spontaneous mutants for lipid metabolism and transgenic rabbits. Though most molecular investigations of the cardiovascular system have used transgenic mice, the small rodents do not accurately reflect crucial facets of human cardiovascular physiology, therefore a number of different transgenic rabbit models of hypertrophic cardiomyopathy were created. Transgenic rabbits have been found to be suitable bioreactors for the production of pharmaceutical proteins filling an important niche between the laboratory mouse and larger farm mammals. It is the smallest animal that can be used to produce recombinant proteins in its milk or serum both on an experimental and a commercial scale. The rabbit appears particularly flexible for the preparation of human antibodies, and recombinant human proteins for replacement therapies have also been produced in rabbit milk. A specific biotechnology of the rabbit is emerging. The scientific community which uses rabbits as experimental animals or as a tool to produce biotech products, as well as those involved in breeding, are invited to focus their efforts on this species.

Key words: rabbit, human disease model, bioreactor, vaccines.

INTRODUCTION

The rabbit is a standard laboratory animal in biomedical research and transgenic rabbits are used as animal models for a variety of human diseases both genetic and acquired. Classical experimental use of rabbits includes antibody production, development of new surgical techniques, physiology and toxicity studies for the testing of new drugs.

The rabbit (*Oryctolagus cuniculus*) is phylogenetically closer to primates than are rodents (Graur *et al.*, 1996) and is large enough to permit non-lethal monitoring of physiological changes. For these reasons, a number of research groups have chosen transgenic rabbits as animal models for the study of lipoprotein metabolism, atherosclerosis, cardiovascular research and hypertrophic cardiomyopathy.

Transgenic rabbits have been found to be suitable bioreactors for the production of pharmaceutical proteins (Houdebine, 1995). The transgenic rabbit system is especially valuable because it fills an important niche between the laboratory mouse and larger farm mammals. It is the smallest animal that can be used to produce recombinant proteins in its milk or serum, both on an experimental and a commercial scale.

The first transgenic rabbits were obtained two decades ago by pronuclear microinjection (Brem *et al.*, 1985; Hammer, 1985). Since then, improvements in the methodology have been reported (Viglietta

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et al., 1997, Besenfelder *et al.*, 1998). This chapter summarises the specific advantages of rabbit models, the accomplishments and future perspectives for their utilisation in biomedical research.

RABBITS FOR HUMAN DISEASE MODELS

Rabbit strains have a more diverse genetic background than inbred and outbred mouse strains. This might be an advantage when studying complex disease models such as atherosclerosis or developing therapeutic strategies, since this situation mimics human genetic diversity more accurately. However, this could also be a drawback and even hamper the use of rabbits in studying the effects of gain or loss of function or in elucidating the mechanism of single gene related diseases. Though transgenic mice have become the most widely used model for human diseases, it is accepted in certain cases that the causative mutations resulting in human diseases do not give rise to the expected pathological symptoms in rodents. For example, the molecular composition of cardiac sarcomeric proteins, and therefore the mutant phenotypes, are more similar to human counterparts in rabbits than in rodent models. Moreover, a number of analytical techniques which are used to study the effects of genetic modifications in mice are limited to a single time point, since the mouse often does not survive the analysis.

Lipid metabolism and atherosclerosis

Rabbits possess several characteristics that made them the first and classical model for the study of lipoproteins and atherosclerosis. Atherosclerosis is a multifactorial disease and is one of the major causes of mortality in Western societies. Rabbit models include normal cholesterol-fed rabbits, spontaneous mutants for lipid metabolism and transgenic rabbits.

Normal rabbit strains in the study of atherosclerosis

Cholesterol fed rabbits are the classical model for studying lipid metabolism and its role in atherosclerosis, as the rabbit is extremely sensitive to a cholesterol-rich diet. Genetically defined JAX rabbits were tested for their serum cholesterol levels in response to a cholesterol-rich diet. Two rabbit strains showing five fold differences in serum cholesterol levels and different sensitivity to cholesterol have been established (Van Zutphen *et al.*, 1977). These two strains, the high responding AX/J and low responding IIIVO/J, were inbred for several generations. AX/JU and IIIVO/JU have been used for genetic analysis of quantitative traits related to dietary cholesterol susceptibility. Application of the AFLP technique with 15 primer combinations revealed 226 polymorphisms between the two inbred strains (Van Haeringen *et al.*, 2002). Recent data point to the fact that dietary cholesterol induces neuronal accumulation of Alzheimer's like beta-amyloid in New Zealand White rabbits, and copper supplementation in drinking water resulted in fibrillar type beta-amyloid accumulation (Sparks, 2004).

Spontaneous mutant rabbit models of human lipoprotein disorders

Hypercholesterolemia is the most important factor in the development of atherosclerosis in humans and experimental animals. In the plasma, three kinds of atherogenic lipoproteins have been identified: low density lipoproteins (LDL) remnant lipoprotein-like β -very low density lipoprotein (β -VLDL), intermediate density lipoproteins (IDL), lipoprotein a Lp(a) and high density lipoproteins (HDL).

Watanabe heritable hyperlipidemic (WHLL) rabbits are well characterised mutants. They are deficient in LDL receptor due to a spontaneously arising deletion in exon 4 of the LDL receptor gene that encodes for a four amino acid deletion in the cysteine-rich ligand-binding domain of the protein (Yamamoto *et al.*, 1986). Homozygous WHLL rabbits are hypercholesterolemic from birth and exhibit

tendon xanthoma and atherosclerosis on chow diet (Watanabe, 1980). The pathology of WHLL rabbits resembles of familial hypercholesterolemia.

The St. Thomas rabbits have increased levels of VLDL, IDL and LDL, due to overproduction of these lipoproteins, and develop atherosclerotic lesions on a chow diet (LaVille *et al.*, 1987). The St Thomas Hospital rabbit strain is thus a potential model for familiar combined hyperlipidemia.

Transgenic rabbits for the study of atherosclerosis

Compared with the most widely used transgenic model, which is the mouse, rabbits have different lipoprotein metabolism features, as summarised in Table 1. Ideally the animal models should share the most important characteristics of the disease with man, which is the case with rabbits. The first transgenic rabbits used to study lipid metabolism by overexpressing the human hepatic lipase and the human apolipoprotein A1 genes were obtained in 1994 (Fan *et al.*, 1994; Duverger *et al.*, 1994). Since then, an increasing number of genetically engineered rabbits have become tools in exploring the function of the proteins associated with dyslipidemia and atherosclerosis (Table 2).

To date, more than a dozen transgenes have been expressed in transgenic rabbits. Some of these animals were naturally mated with each other or with WHLL rabbits to create double mutant animals. The phenotypic properties of transgenic rabbit models in the study of atherosclerosis and dyslipidemia have been described (Brousseau and Hoeg, 1999; Fan *et al.*, 1999; Fan and Watanabe, 2000a; 2003).

In some cases, several genes were introduced simultaneously. This situation was encountered for the rabbits harbouring the human apoA1-apoCIII-apoAIV gene cluster, which allows a coordinated expression of the three genes (Recalde *et al* 2004). Other transgenic rabbits not described here are also used to study lipid metabolism and obesity (unpublished data).

These animals are being used to evaluate the effects of new drugs which stimulate human genes and are expected to protect patients against atherosclerosis. These rabbits were fed with a cholesterol-rich diet. It was not possible to determine with certainty whether these animals were protected against atherosclerosis. Indeed, the surface of the plaques in the aorta was very different in the animals, regardless of whether the animals were transgenic or not (Recalde *et al.*, 2004). This problem was clearly due to the broad genetic heterogeneity of the rabbits, which is a factor that frequently reduces the relevance of rabbit models in the study of atherosclerosis.

Table 1: Lipoprotein metabolism features of rabbits.

	Mouse	Rabbit	Human
Lipoprotein profile	HDL-rich	LDL-rich	LDL-rich
CETP	No	Yes	Yes
Hepatic apoB editing	Yes	No	No
apoB48	Chylomicron VLDL	Chylomicron	Chylomicron
Hepatic lipase activity	High, 70% in circulation	Low, liver-bound	High, liver-bound
Hepatic LDL receptor	Usually high	Down-regulated	Down-regulated
ApoA-II	Yes	No	Yes
Dietary cholesterol	Resistant (most strains)	Sensitive	-
Atherosclerosis	Resistant	Susceptible	-

Table 2: Application of transgenic rabbit models in lipid metabolism and atherosclerosis.

Expressed human gene	Main results	Reference(s)
Human hepatic lipase	Key role in plasma cholesterol homeostasis. Elevated hepatic lipase levels inhibited diet-induced atherosclerosis.	Fan <i>et al.</i> (1994)
Human lipoprotein lipase	Reduced total cholesterol and atherogenic very low density lipoprotein levels. 80% decrease in plasma triglycerides 59% decrease in high density lipoprotein-cholesterol	Araki <i>et al.</i> (2000) Fan <i>et al.</i> (2001)
Human 15-lipoxygenase	Protection against atherosclerosis development.	Shen <i>et al.</i> (1995)
Human lecithin:cholesterol acyltransferase	Significantly less aortic atherosclerosis.	Hoeg <i>et al.</i> (1996)
Human apo A-I	Inhibition of atherosclerosis, impaired vasorelaxation. Isolated increase in high density cholesterol levels not effective in inhibiting atherosclerosis. Pro-atherosclerotic diet equally reduced paroxonase activity in control and apo A-I transgenic rabbits.	Duverger <i>et al.</i> (1996a, b) Lebuffé <i>et al.</i> (1997) Boullier <i>et al.</i> (2001) Mackness <i>et al.</i> (2000)
Human apo A	Covalent association with rabbit apo B, new insights into Lp(a) assembly. Extensive atherosclerosis in apo A transgenic vs. normal rabbits in response to cholesterol rich diet.	Rouy <i>et al.</i> (1998) Fan <i>et al.</i> (2000) Fan and Watanabe (2000b)
Human apo B-100	Overexpression of apo B resulted increased levels of the atherogenic low density lipoprotein (LDL) and decreased levels of the anti-atherogenic high density lipoproteins (HDL).	Fan <i>et al.</i> (1995)
Human apoA-I/C-III/A-IV gene cluster	Higher plasma total cholesterol, HDL-cholesterol and total phospholipid concentrations.	Recalde <i>et al.</i> (2004)
Human apo E variants	Animal model of type III hyperlipoproteinemia, points to the role of sex hormones. Reduced levels of very low density lipoprotein, accumulation of low density lipoprotein.	Huang <i>et al.</i> (1997) Fan <i>et al.</i> (1998)
Macrophage derived Human matrix metalloproteinase 12	Animal model for mechanistic studies in inflammatory diseases and cancer invasion. Enhanced arthritic lesions and cartilage degeneration.	Fan <i>et al.</i> (2004) Wang <i>et al.</i> (2004)
Macrophage derived Lipoprotein lipase	Macrophage derived lipoprotein lipase is proatherogenic.	Ichikawa <i>et al.</i> (2005)
Rabbit apo B mRNA editing enzyme APOBEC-1 component 1 (APOBEC-1)	Liver dysplasia in all transgenic rabbit lines => not applicable for gene therapy. Due to low expression levels apo B mRNA is not edited. The distinct CpG methylation pattern of the rat proximal promoter is lost in the transgenic rabbit	Yamanaka <i>et al.</i> (1995) Apostel <i>et al.</i> (2002)

On the other hand, in a number of cases transgenic rabbits showed different phenotypes from transgenic mice, even though the same genes were introduced and the phenotype of transgenic rabbits was closer to humans (Fan and Watanabe, 2003). This again underlines the importance of choosing an appropriate species to be used in transgenic investigation.

Hypertrophic cardiomyopathy

In vivo models are invaluable to provide integrative data regarding physiological and pathological states of the heart, such as cardiac hypertrophy and dilation. Though most molecular investigations of the cardiovascular system have used transgenic mice, the small rodents do not accurately reflect the crucial facets of human cardiovascular physiology. Hypertrophic cardiomyopathy (HCM) is a relatively common disease that is diagnosed by the presence of left ventricular hypertrophy in the absence of an increased external load. Mutations in eight genes encoding sarcomeric proteins have been identified in patients with HCM. Transgenic mice expressing a variety of mutant sarcomeric proteins did not reiterate the hallmark of HCM in humans: left ventricular hypertrophy. On the other hand, a transgenic HCM rabbit model created by expressing a common point mutation in human β -myosin heavy chain (Q403) revealed all the characteristic symptoms observed in human patients (Marian *et al.*, 1999). Prior to the clinical trials, simvastatin was applied to β -myosin heavy chain-Q403 transgenic rabbits to test the hypothesis that hypertrophy and fibrosis, the major determinants of mortality in HCM patients, are reversible by simvastatin treatment (Patel *et al.*, 2001). Atorvastatin, which is known to block hypertrophic signaling, was also found effective on the β -myosin heavy chain-Q403 transgenic rabbits, and is pending clinical trials (Senthil *et al.*, 2005).

Another aspect of hypertrophic cardiomyopathy for which transgenic rabbits are the most appropriate model is Ca^{2+} handling during contraction/relaxation and alterations in Ca^{2+} influx during heart failure. Cardiac troponin mutations can cause familial HCM. Troponin mutations expressed in transgenic mouse heart showed that these animals were able to tolerate only minor amounts of the mutant protein before dosage became lethal (Tardiff *et al.*, 1998). This reflects the differences in heart rates and cardiac cycles between the two species. Rabbits have a slower heart rate, and recently an inhibitory domain mutation was mimicked by expressing the mutant protein in the rabbit ventricle. A modest amount of the mutant protein caused subtle defects without seriously affecting cardiac function (Sanbe *et al.*, 2005).

Susceptibility to tuberculosis

Tuberculosis (TB) remains a leading cause of infectious disease mortality, causing two million deaths yearly. Because of the complex TB pathogenesis, the development of animal models for specific stages of the disease, such as latency and cavitation, remains a priority. The rabbit model offers certain advantages over both the murine and guinea pig models. Historically, the rabbit model has been used to differentiate *Mycobacterium tuberculosis* from *Mycobacterium bovis*, based on their relative virulence in this animal host. Rabbits develop a disease that is similar to TB in humans, namely granulomas with caseous necrosis, liquefaction and cavities. Specific pathogen free (SPF) New Zealand White (NZW) rabbits were used to evaluate the virulence of three *Mycobacterium tuberculosis* strains and to identify stage specific *Mycobacterium tuberculosis* genes important in the human disease (Manabe *et al.*, 2003). Recently published data describe an inbred SPF NZW rabbit strain which is more susceptible to TB than the outbred counterparts due to cell mediated immune defects (Dorman *et al.*, 2004).

AIDS, cancer and spongiform encephalopathy studies

Animal models are essential for understanding the pathogenesis of retrovirus-induced immune deficiency and encephalopathy and for development and testing of new therapies and vaccines. AIDS and related disorders are etiologically linked to various members of the lentivirus subfamily of retroviruses. These lymphocytopathic lentiviruses are designated human immuno-deficiency virus type 1 (HIV-1) and human immuno-deficiency virus type 2 (HIV-2). The only animals susceptible to experimental HIV-1 infection are the chimpanzee, the gibbon ape, and the rabbit. The progression of HIV-1 infection in laboratory rabbits is very slow due to critical differences between human and rabbit CD4 binding sites for viral protein HIV gp120. To tentatively improve this model, transgenic rabbits that express the human CD4 gene were created (Snyder *et al.*, 1995, Dunn *et al.*, 1995). *In vitro* data on transgenic rabbit lymphocytes revealed the requirement for huCD4 in initiation, but not the spread of HIV-1-induced apoptosis (Leno *et al.*, 1995). *In vivo* infection of huCD4-transgenic rabbits using HIV-1III_B-infected autologous lymphocytes was demonstrated by virus isolation, detection of HIV-1-specific DNA in peripheral blood lymphocytes and seroconversion to various HIV-1 proteins (Dunn *et al.*, 1995). In spite of the promising results obtained with the huCD4 transgenic rabbits, which were created by two independent laboratories, no additional data based on utilisation of the huCD4 transgenic rabbits were published. It would have been interesting to sensitize the CD4 rabbits to HIV by adding the human CCR5 gene, which is the second essential component of HIV receptor. These experiments were not carried out mainly because rabbit handling at level 3 confinement is too costly.

Oncogene expressing transgenic rabbits, which developed lymphomas (Knight *et al.*, 1988, Sethupathi *et al.*, 1994) and skin carcinoma (Peng *et al.*, 1993; 1995; 1999; 2001) were created and partially characterised. Those animal models may be valuable for studying oncogenes and their synergistic effects in tumorigenesis. Furthermore they provide a model for evaluating antitumor therapies (reviewed by Fan and Watanabe, 2003; Bösze *et al.*, 2003).

Transgenic rabbits expressing the ovine PrP gene have been obtained. These animals, but not the non-transgenic control, proved highly sensitive to infection by scrapie (unpublished data). These rabbits are useful models for deciphering the mechanisms of prion infection.

Anatomical and metabolic disorders

Acromegaly is a chronic disease in which prolonged elevation of growth hormone (GH) levels after puberty leads to overgrowth of the skull and of the extremities. Glucose intolerance and insulin resistance is manifested in about 60% of the patients (Ganda and Simonson 1993). GH overexpressing transgenic mice had giant phenotype, which is not a relevant model for human acromegaly (Palmiter *et al.*, 1982). Transgenic pigs overexpressing GH did not show typical acromegalic alterations, but developed arthritis and ulcers (Pinkert *et al.*, 1994). On the other hand, transgenic rabbits expressing GH gene reiterated the skeletal abnormalities, the metabolic differences and the histopathological alterations in the liver and muscle of humans suffering from acromegaly (Costa *et al.*, 1998).

Surgically induced secondary osteoarthritis and osteotomy rabbit models have also been created for *in vivo* gene therapy trials (Rogart *et al.*, 1999, Bertone *et al.*, 2004). In the osteoarthritis model, the interleukin-1 receptor antagonist gene was found to be effective in the prevention of osteoarthritis progression (Fernandes *et al.*, 1999). In the osteotomy model the gene therapy vector harboured the human BMP-6 gene and was shown to accelerate healing (Bertone *et al.*, 2004).

Transgenic rabbit organs expressing human DAF and CD59 genes in all their cells are resistant to complement attack (Taboit-Dameron *et al.*, 1999).

Use of GFP rabbits

GFP (green fluorescent protein) is a protein from Pacific Ocean jellyfish which becomes green under UV light. It is increasingly used as a marker to follow cells expressing the GFP gene. One advantage of this protein is that its fluorescence needs no substrate and that repeated non-invasive observations can be performed *in vivo* on the same animal.

Various transgenic species, including rabbits, harbouring the GFP gene have been obtained. The human eF1- α gene promoter was used to drive the expression of GFP gene in all cell types of the rabbits (Boulangier *et al.*, 2002).

The observation of the GFP rabbit cells using a specific probe (Cell-viZio, MKT) made it possible to obtain the precise description of different tissues which appear green (Al Gubory and Houdebine, unpublished data).

GFP rabbits are currently being used as models to study cornea grafting. These rabbits made following the fate of the green cells possible at different periods after cornea grafting on normal rabbits (Cardiatikis-Myers *et al.*, unpublished data).

TRANSGENIC RABBITS AS BIOREACTORS

Pharmaceutical proteins produced from transgenic rabbit milk

The technology for using the mammary gland as a bioreactor has been developed to the point that pharmaceuticals derived from the milk of transgenic farm animals are currently in the advanced stages of clinical trials. It is generally admitted that transgenic organisms may be the source of abundant and cheap therapeutic proteins. Transgenic plants suffer from the fact that they do not glycosylate proteins as animal cells do. Moreover, no dissemination of transgenic plants synthesizing pharmaceutical proteins should occur. Animals do not raise these two problems (Houdebine, 2002).

The time required to generate transgenic animals with high expression levels and to deliver a product to the market are the major drawbacks to large animal transgenic technology. Transgenic rabbits are an attractive alternative to large dairy animals because of their large litter size and short generation interval (Houdebine, 1995; Dove, 2000). Rabbits are easily milked and their milk composition is well characterised (Baranyi *et al.* 1995). Rabbit milk naturally contains 2.5 and 4.8 times as much protein as sheep and goat milk respectively (Jennes *et al.*, 1994). The transgenic rabbit is a very efficient system for testing new gene constructs and has the advantage over mice that sufficient protein can be produced for activity testing and even for clinical trials of therapeutic proteins (Van den Hout *et al.*, 2001, 2004). Pharmaceutical products which were produced in transgenic rabbit milk can be divided into three categories: (a) hormones and bioactive peptides, (b) therapeutic proteins and (c) vaccines. Table 3 shows the hormones and bioactive peptides which have been produced in transgenic rabbit milk to date and indicates the observed side-effects of the recombinant proteins on animals. On the other hand, it should be considered that one of the theoretical concerns, and the reason for the moderate public acceptance in some countries for the production of bioactive hormones from the milk of transgenic animals, is their potential deleterious side-effects on reproducing-females and their litters.

Recombinant human proteins for replacement therapies have also been produced in rabbit milk. To date, production of human C1 inhibitor, alpha-glucosidase and the blood clotting factor VIII were reported (Bijvoet *et al.*, 1999, Hiripi *et al.*, 2003, Koles *et al.*, 2004a). Among these proteins, the human C1 inhibitor and alpha-glucosidase reached the phase III clinical trials (<http://www.pharming.com/>, Van den Hout, 2004).

Table 3: Hormones and bioactive peptides produced in the milk of transgenic rabbits.

Expressed protein	Gene	Maximal expression levels Other findings/side-effects	Reference(s)
Interleukin 2 (IL29)	Rabbit β -casein-genomic DNA	0.0005 g/l	Buhler <i>et al.</i> , 1990
Human growth hormone	Mouse whey acidic protein-genomic DNA	0.05 g/l. hGH in the serum.	Limonta <i>et al.</i> , 1995
Human insulin-like growth factor	Bovine α s1-casein expression cassette-cDNA	1 g/l. Upregulation of IGFb-2, increased milk yield.	Brem <i>et al.</i> , 1994; Wolf <i>et al.</i> , 1999; Znovieva <i>et al.</i> , 1998
Bovine chymosin	Bovine α s1-casein expression cassette prochymosin gene	10 g/l. Healthy animals and no unwanted clotting.	Brem <i>et al.</i> , 1995
Erythropoietin	Rabbit whey acidic protein-cDNA	0.0003 g/l	Rodriguez <i>et al.</i> , 1995
Erythropoietin	Rabbit whey acidic protein-cDNA and genomic DNA	0.05 g/l. High red blood cell counts, sterility, agalactia and premature death in one transgenic line.	Massoud <i>et al.</i> , 1996
EC superoxide dismutase	Mouse whey acidic protein-cDNA EC SOD	3 g/l. Not fully glycosylated, biologically active.	Strömqvist <i>et al.</i> , 1996
Erythropoietin	Bovine β -lactoglobulin-cDNA (fusion protein of erythropoietin with β -lactoglobulin to avoid biological activity in the secreting animal)	0.05 g/l. Correct glycosylation, transient increase in hematocrit values of lactating females.	Korhonen <i>et al.</i> , 1997
Salmon calcitonin	Ovine β -lactoglobulin-cDNA (fusion protein of salmon calcitonin with human -lactoglobulin)	2.1 g/l. First evidence of C-terminal amidation by the mammary gland.	McKee <i>et al.</i> , 1998
Human nerve growth factor β	Bovine α s1-casein expression cassette-genomic DNA.	0.25 g/l. Equivalent biological activity with the commercial product.	Coulbaly <i>et al.</i> , 1999
Human acid α -glucosidase	Bovine α s1-casein-genomi DNA.	8.0 g/l. Pompe disease treatment.	Bijvoet <i>et al.</i> , 1999
Equine chorionic gonadotropin	Rabbit whey acidic protein-single $\beta\alpha$ chain.	0.022 g/l. Rapid plasma clearance no <i>in vivo</i> biological activity.	Galet <i>et al.</i> , 2001
Bovine follicle stimulating hormone	Bovine α s1-casein expression cassette-cDNA for α and β chains	0.005 g/l. Two double transgenic rabbit lines, full <i>in vitro</i> biological activity.	Coulbaly <i>et al.</i> , 2002
Human C1 inhibitor	Unpublished (Heus <i>et al.</i>)	12 g/l. Replacement therapy in hereditary angioedema.	Koles <i>et al.</i> , 2004a; b
Rotavirus VP2 and VP6 as vaccine	Rabbit whey acidic protein-cDNA	0.5 g/l. Protects mice against rotavirus.	Soler <i>et al.</i> , 2005a; b

Though the number of applications is steadily increasing, it should be pointed out that the commercial success of recombinant hormones, bioactive peptides, therapeutic proteins or vaccines which are in competition with alternative methods of production has still not occurred. One of the reasons is that proteins are naturally very complex molecules and that transgenic animals are a new method of producing these pharmaceuticals. This means that the pioneer molecules obtained in this way are very closely examined before being released on the market.

A key point in the production of pharmaceuticals by recombinant organisms is the posttranslational modifications of the proteins. In comparison with bacteria, plant or animal cells culture production systems, little is known about the glycosylation machinery of the mammary gland, and thus about the glycosylation of recombinant glycoproteins produced in transgenic animals. Recent studies of species-specificity in the glycosylation of proteins such as IgG underscore the importance of choosing the appropriate species for producing fully functional recombinant proteins (Raju *et al.*, 2000). Rate limitation of the γ -carboxylation of human protein C in the mammary glands of transgenic mice and pigs suggested that it is necessary to perform similar tests with recombinant proteins produced in rabbit milk (Subramanian *et al.*, 1996). Detailed characterization of the N and O-glycans of recombinant human C1 inhibitor (rhC1I) from transgenic rabbit milk has been performed recently (Koles *et al.*, 2004 a; b). The major N-glycan structures of rhC1I from pooled rabbit milk were essentially similar to those of native human C1 inhibitor and recombinant human C1 inhibitor produced in transgenic mouse milk. Only the degree of sialylation and core fucosylation was lower in rabbit milk than in the native protein. When large quantities of rhC1I were isolated for preclinical and clinical studies, highly consistent N-linked glycan profiles and monosaccharide compositions were found (Koles *et al.*, 2004b).

On the other hand, ruminants are known to add NGNA (N-glycosyl neuraminic acid) as well as NANA (N-acetyl neuraminic acid) to proteins (Edmunds *et al.*, 1998). Interestingly, rhC1I produced in rabbit milk contained essentially NANA (Koles *et al.*, 2004b). From this point of view, rabbits appear to be a better bioreactor than other species.

Monoclonal antibodies

Monoclonal antibodies have been produced by the microinjection of constructs containing cloned genes for the light and heavy chains of mouse monoclonal antibodies. Two transgenic rabbit founders obtained in this way showed antibody serum levels of 150-300 $\mu\text{g/ml}$. Their offspring consistently exhibited a more elevated expression level: 150mg/ml. The purified antibody was shown to have intact binding sites for the antigen but isoelectric focusing revealed that only a small fraction of the transgenic antibody was actually identical with the mouse monoclonal antibody (Weidle *et al.*, 1991). This discrepancy was attributed to formation of heterologous antibodies and to species-specific posttranslational modifications. Indeed, species-specific IgG glycosylation has been reported by Raju *et al.* (2000).

The creation of *c-myc* and *v-abl* transgenic rabbits made it possible to obtain rabbit plasmacytoma cell lines and create rabbit-rabbit hybridomas producing rabbit monoclonal antibodies (Spieker-Polet *et al.*, 1995). This offers the possibility of producing rabbit monoclonal antibodies which may be further humanised and produced in the milk of transgenic animals as recombinant proteins. For unknown reasons, the rabbit plasmacytoma cell lines are not used extensively to obtain rabbit monoclonal antibodies.

Artificial bispecific single chain antibodies which redirect immunologic effector cells towards tumor cells have also been produced recently in transgenic rabbits (Goose-Hovest *et al.*, 2004). The

bispecific protein, which was purified from the serum of the transgenic rabbits was directed to a melanoma-associated proteoglycan and the human CD28 molecule. It was fully active and mediated target-cell restricted T cell stimulation and tumor cell killing.

Polyclonal antibodies

Though monoclonal human-like antibodies currently represent a market of \$9 billion yearly sales, they react with specific targets, which may change, evolve and mutate during the spread of disease throughout a population or within an individual. Polyclonal antibodies are highly potent against multiple antigenic targets, can neutralize toxins and direct immune responses to eliminate pathogens. Since rabbits have been used for many decades for the commercial production of polyclonal antibodies, an early-stage biopharmaceutical company, the Therapeutic Human Polyclonals Inc (<http://www.polyclonals.com/>) started developing transgenic rabbits producing human polyclonal antibodies after conventional immunisation of the animals with the antigens of interest. The proof of principle experiments was presented recently by Buelow (2006). The same project is being developed also in mice, pigs and cows. It should be mentioned that trans-chromosomal calves carrying the entire human immunoglobulin (hIg) heavy-chain (H) and lambda (lambda) light-chain loci represent another transgenic livestock species to produce polyclonal human antibodies (Kuroiwa *et al.*, 2002).

To obtain fully human polyclonal antibodies, the human loci containing the gene coding for antibodies must be introduced into the genome of the animals, which must also have lost their own immunoglobulin genes. This can be achieved in mice by knocking out immunoglobulin genes in ES cells and generating chimaeric animals harbouring the mutation (Ishida *et al.*, 2002). In other species, gene knockout must be performed in somatic cells further used to generate cloned mutated animals (Kuroiwa *et al.*, 2002).

Available rabbit strains are naturally devoid of several immunoglobulin loci. These animals, which received human immunoglobulin loci, generate human polyclonal antibodies after classical immunisation (Buelow, 2006). The rabbit appears to be particularly flexible in the preparation of human antibodies.

Vaccines

Conventional vaccines rely on the use of attenuated pathogens. These vaccines are often efficient but not always fully safe and not easily prepared in large quantities. Recombinant pathogen proteins appear to be getting safer and more easily available than classical vaccines.

Associated viral proteins forming VLPs (virus like particles) having strong immunogenic activities and being devoid of nucleic acids proved to be efficient vaccines in several cases. VLPs containing VP2 and VP6 rotavirus proteins have been obtained in rabbit milk at a concentration as high as 500µg/ml (<http://www.bioprotein.com>). Milk fractions administered in mice protected 100% of the animals against an infection by rotavirus (Soler *et al.*, 2005; 2006)

CONCLUSIONS

The experiments described in this paper and others emphasize the specific importance of the rabbits as models and protein producers. Rabbits are also a significant source of meat in different countries. This supports the idea that the rabbit will be more widely used for specific biotechnology projects. Transgenesis is presently a limited but classical tool in this species. Cloning has been successfully achieved, although this technique must be improved to be really utilisable in creating valuable models. Rabbit genome sequencing is in the course of providing researchers with unknown genes

and genetic markers. A clear limitation for the use of rabbits as models in some cases is their extreme genetic diversity. The establishment of one or two lines of inbred rabbits having more homogeneous biological properties would be very useful.

A specific biotechnology of the rabbit is therefore emerging. The first international meeting on transgenic rabbits took place in Tsukuba (Japan) in 2005. Another meeting on "Use of rabbits as models of infectious diseases in humans" was also held in 2005 at the NIH, USA. The scientific community which uses rabbits as experimental animals or as a tool for the production of biotech products, as well as those involved in breeding, would be well advised to focus their efforts on this species.

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