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GENERAL TOPIC: APPLICATIONS OF TRANSGENIC RABBITS IN BIOMEDICAL RESEARCH - BASED ON LITERATURE SEARCH

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Abstract: Transgenic rabbits are widely used as a model organism for biomedical research, and the transgenic rabbit system is especially valuable because it fills an important niche between laboratory mice and larger domesticated mammals. In order to describe the current status and development trends of the use of transgenic rabbits in biomedical research precisely, we performed a quantitative analysis of the published data, collected by searching biomedical databases. Currently, there are about 217 papers related to transgenic rabbits, originating from 22 countries. The number of publications has slowly increased over time, reaching its peak in 2004 and 2007. Approximately one third of the publications come from the USA, and one quarter come from Japan. The USA, Japan and France were the top three producers of publications related to transgenic rabbits. These publications mainly focused on cardiovascular disease (CVD) and the study of therapeutic protein bioreactors. Approximately 19 transgenic rabbit lines have been established for the study of CVD, and 20 recombinant proteins have been produced from transgenic rabbit milk or blood. The remaining publications largely focused on virology and immunology, diabetes mellitus, cancer, and genetics. These publications provide new insights into the mechanisms responsible for the development of human disease and shed light on the management of some genetic disorders. Thus, this quantitative review of the literature reveals that transgenic rabbits play an increasingly important role in biomedical research.

Key Words: transgenic rabbits, biomedical research, atherosclerosis, bioreactor.

INTRODUCTION

Investigating the biological functions of genes and proteins and their relationship with the pathogenesis of human diseases requires appropriate and relevant animal models. Although mice (wild type and genetically modified) offer many research avenues, other non-murine species, such as rabbits, are required in some studies. The first reports on the application of transgenic technology in the rabbit were given by Hammer *et al*. (1985), who performed an initial trial to express human growth hormone under the control of the mouse metallothionein promoter. During the last decade, much progress has been made in rabbit biotechnology, including the achievement of rabbit transgenesis, cloning of rabbits, rabbit ES cells, and sequencing of the rabbit genome; hence, a specific branch of biotechnology related to the rabbit

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is emerging. This organism is genetically and physiologically close to humans and has been extensively used in many aspects of biological studies and medical research (Fan *et al*., 2003; Houdebine, 2009; Bosze *et al*., 2003, 2006; Brousseau *et al*., 1999; Taylor *et al*.1997). The transgenic rabbit system is especially valuable because it fills an important niche between laboratory mice and larger domesticated mammals. The rabbit is the smallest domesticated animal that can be used for the production of recombinant proteins from its milk on both experimental and commercial scales (Bosze *et al*., 2003). Because of their moderate size, rabbits are easier to manipulate in experiments such as those in which blood samples are drawn. Several characteristics of the rabbit also make it an excellent model in which to study human diseases such as atherosclerosis. After twenty five years of development, transgenic rabbits have been widely accepted by scientists as an excellent animal for biomedical research. Here, we performed a quantitative analysis on the current status of transgenic rabbits in biomedical research by collecting published data through searching the PubMed, SCI (Science Citation Index Expanded), and EMBASE (Elsevier) databases.

MATERIALS AND METHODS

Selection of databases

Medline is the largest biomedical database in the world and is the most commonly used search engine (Ebbert *et al*., 2003). PubMed is the internet version of Medline (www.ncbi.nlm.nih.gov/), containing bibliographic citations and author abstracts from more than 4,800 biomedical journals published in the USA and 70 other countries. PubMed was selected as the main database to retrieve the publications related to transgenic rabbits. In order to include more publications in this study, the search results of SCI (Science Citation Index Expanded) and EMBASE (Elsevier) were included as a complimentary approach.

Search strategy

PubMed, EMBASE, and SCI were searched comprehensively to identify publications related to transgenic rabbits published from 1966 onwards in the biomedical field. The keywords used in the literature searches included: transgenic rabbits, transgenic rabbit, transgene, and transgenic animal (2009-04-12).

Publication inclusion criteria

The inclusion criteria applied were (i) article or review, (ii) studies that used or established transgenic rabbit models or developed new techniques for creating transgenic rabbits, (iii) language of publication – any language. Duplicated studies were excluded by examining the author list, parent institution, sample size, and study results.

Data extraction

Data were independently extracted for quantitative analysis by two authors from publications that met the inclusion criteria, and any disagreement was subsequently resolved by discussion. The quantitative data included the following: what kind of transgenic rabbits were used; publication names and issues; reprint authors, published date; the number of times a publication was cited; journal impact factors.

Quantitative analysis

A quantitative analysis of the publications related with transgenic rabbits was then performed on the extracted data. The outcome measure included (i) the variance of publications related to transgenic rabbits from 1998 to 2008, (ii) the distribution of publications related to transgenic rabbits with respect to different countries, journals, research areas and research teams, (iii) the highest impact factor and top cited papers, (iv) the kinds of transgenic rabbits used in biomedical research.

RESULTS

Literature search

An electronic database search performed on March 15, 2009, yielded 223, 262 and 158 publications in PubMed, SCI, and EMBASE, respectively. After two researchers checked each of these publications and excluded duplicated publications, meeting abstracts, and comments, 217 publications (articles, 198; reviews, 19) that matched the selection criteria remained. There was unanimity between the two researchers regarding the selection of relevant publications.

Annual variance of publications related to transgenic rabbits

Since the first transgenic rabbits were generated approximately two decades ago by pronuclear microinjection, the number of papers related to transgenic rabbits published increased slowly every year, reaching 12 in 1995. The number of publications on transgenic rabbits per year ranged from 7 to 20 from 1995 to 2008 and reached its peak during 2004 and 2007 (Figure 1).

Distribution of publications related to transgenic rabbits

According to our literature search results, the 217 publications related to transgenic rabbits originated from 22 countries (Figure 2). Approximately one third of the publications came from the USA, while one fourth came from Japan. The USA, Japan, and France were the top three producers of publications related to transgenic rabbits.

All of the publications related to transgenic rabbits were distributed in 94 journals. The top 3 journals, based on impact factor, are Nature, Nature Biotechnology, and the Journal of Clinical Investigation. Among the 217 publications, 16 were published in "Transgenic Research", 9 in "Arteriosclerosis, Thrombosis, and Vascular Biology", 8 in "Circulation", and the rest were published in other journals (Figure 3). The top 3 most cited publications were as follows: Hammer *et al* (1985), published in Nature (cited times: 462); Fan *et al* (1994) and Yamanaka *et al* (1995), both published in Proceedings of the National Academy of Sciences of the United States of America (cited 167 and 130 times, respectively).

We also analysed the research topics of the 198 primary articles related to transgenic rabbits and found that studies related to cardiovascular disease, the use of rabbits as bioreactors for therapeutic protein production, and biotechnological research (for the purpose of improving the technologies used in

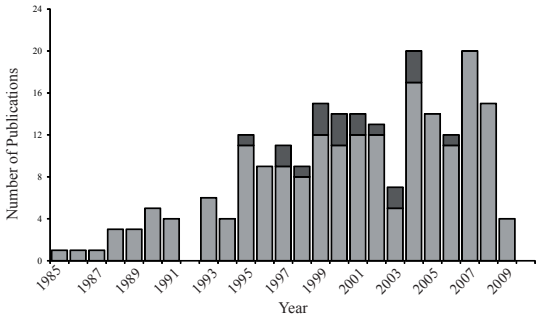

Figure 1: Annual variance of publications (■ review and ■ article) related to transgenic rabbits (cites available at Appendix A, only for electronic version).

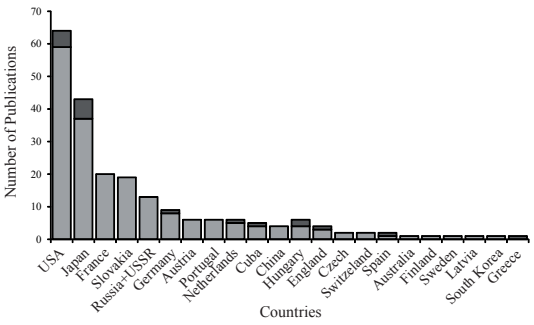

Figure 2: Distribution of publications (■ review and ■ article) related to transgenic rabbits in different countries (cites available at Appendix A, only for electronic version).

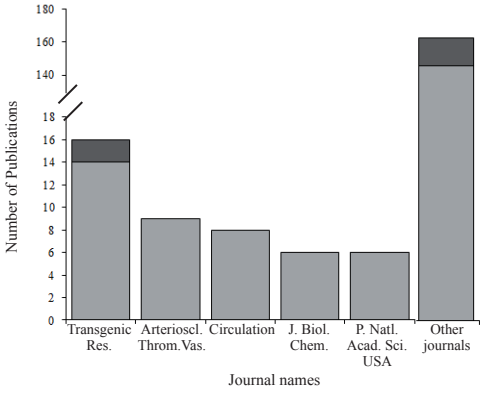

Figure 3: Distribution of publications (■review and ■article) related to transgenic rabbits in different journals (cites available at Appendix A, only for electronic version)..

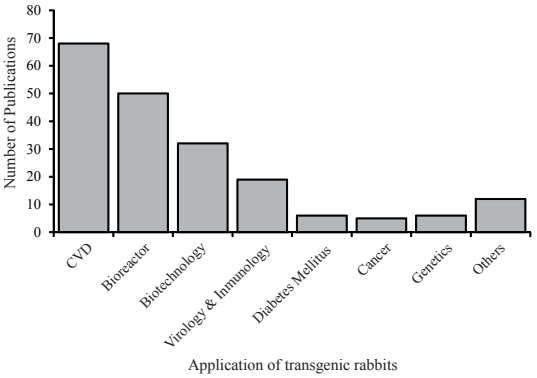

Figure 4: Distribution of publications related to transgenic rabbits in different research subjects (cites available at Appendix A, only for electronic version).

transgenic rabbit methodology) were the three most published upon areas. The remaining articles focused on a range of topics, including virology and immunology, diabetes mellitus, cancer, and genetics (Figure 4).

Currently established transgenic rabbits are used as human cardiovascular disease models and therapeutic protein bioreactors

According to our results, the most important applications of transgenic rabbits are as human disease models for the study of cardiovascular disease (CVD) and as therapeutic protein bioreactors. Approximately 19 transgenic rabbit lines have been established for the study of CVD; the transgenes expressed include apo(a) (Rouy *et al*.,1998; Fan *et al*., 1999a), apoA-I (Duverger *et al*. 1996a, 1996b), apoB-100 (Fan *et al*., 1995), apoE2 (Huang *et al*., 1997), Human apoA-I/C-III/A-IV (Recalde *et al*.,2004), apoE3 (Fan *et al*., 1998), Human hepatic lipase (HL) (Fan *et al*., 1994), lecithin:cholesterol acyltransferase (LCAT) (Hoeg *et al*.,1996), lipoprotein lipase (LPL) (Araki *et al*. 2000), 15-lipoxygenase (15-LO) (Shen *et al*., 1995), matrix metalloproteinase-12 (MMP-12) (Fan *et al*., 2004), apoB mRNA editing enzyme catalytic polypeptide 1 (APOBEC-1) (Yamanaka *et al*.,1995), beta-MyHC-Q(403) (Marian *et al*.,1999), Human genes KCNQ1 and KCNH2 (Brunner *et al*.,2008), phospholamban (Pattison *et al*., 2008), cardiac Gsalpha (Nishizawa *et al*., 2007), vascular endothelial growth factor (165) (Kitajima *et al*., 2005), cardiac troponin I (146Gly) (Sanbe *et al*., 2005), and myosin light chain (M149V) (James *et al*., 2002).

Using an appropriate promoter, approximately 20 recombinant proteins have also been expressed in and isolated from transgenic rabbit milk or blood, as summarised in Table 1. These potentially therapeutic proteins include the following: human α1-antitrypsin, human IL-2, human tPA, human erythropoietin, human insulin-like growth factor-1, human extracellular superoxide dismutase, human GH, human GH releasing factor, human a-glucosidase, salmon calcitonin, equine chorionic gonadotropin (eCG), human nerve growth factor (hNGF-β), human protein C, chymosin, alkaline phosphatase, human C1 inhibitor, human interferon beta, recombinant VP2 and VP6 proteins, low-phenylalanine kappa-casein, and human factor VIII.

Table 1: Therapeutic recombinant proteins produced in transgenic rabbits.

Proteins	Potential therapeutic use	Protein concentration	Promoter	References
Human α1-antitrypsin	Emphysema	1 mg/mL plasma	Human α1-antitrypsin	Massoud *et al*., 1990,1991
Human IL-2	?	~0.43 mg/mL	Rabbit β-casein	Buhler *et al*., 1990
Human tPA	Thrombosis	~50 µg/mL	Bovine αS1-casein	Reigo *et al*., 1993
Human erythropoietin	Anaemia	0.3 ng/mL	Rabbit WAP	Rodriguez *et al*., 1995
		50 µg/mL	Rabbit WAP	Massoud *et al*., 1996;
		0.5 mg/mL	Bovine β-lactoglobulin	Korhonen *et al*., 1997
Human insulin-like growth factor-1	GH deficiency/resistance, DM, osteoporosis, cardiomyopathy	~1 mg/mL	Bovine αS1-casein	Brem *et al*., 1994
		50-300 µg/mL	Bovine αS2-casein	Wolf *et al*., 1997
		543 µg/mL	Bovine αS1-casein	Zinovieva *et al*., 1998
Human extracellular superoxide dismutase	Osteoarthritis, ischemia and post-ischemic reperfusion	3 mg/mL	Mouse WAP	Stromqvist *et al*., 1997
Human GH	GH deficiency	250 ng/mL plasma	Mouse metallothionein-I	Hammer *et al*., 1985
		50 µg/mL	Mouse WAP	Limonta *et al*., 1995
human GH releasing factor	dysfunction of the endocrine glands	?	mouse metallothionein I gene	Koval *et al*., 1991
Human α-glucosidase	Glycogen storage disease	8 mg/mL	Bovine αS1-casein	Bijvoet *et al*., 1999
Salmon calcitonin	Osteoporosis, Paget's disease, and hypercalcaemic shock	~2.1 mg/mL	Ovine β-lactoglobulin	McKee *et al*., 1998
Equine chorionic gonadotropin (eCG)	?	27.1 µg/mL	Rabbit WAP	Galet *et al*., 2000
Human nerve growth factor (hNGF-β)	Neuropathy	50-250 µg/mL	Bovine αS1-casein	Coulibaly *et al*., 1999
Human protein C	hPC deficiency	0.109-0.301µg/mL	Mouse WAP	Dragin *et al*., 2005
Chymosin	Cheese production	0.5-2 mg/mL	Bovine αS1-casein	Brem *et al*., 1995
Alkaline phosphatase	Gram-negative bacterial lipopolysaccharide (LPS) mediated acute and chronic diseases	826 U/ mL	Mouse WAP	Bodrogi *et al*., 2006
Human C1 inhibitor	Hereditary angioedema		?	Koles *et al*., 2004
Human interferon beta	Anti-viral protein	2.2-7.2 ×104 IU/mL	Sheep beta lactoglobulin	Khodarovich *et al*., 2008
Recombinant VP2 and VP6 proteins	Infantile viral gastroenteritis	50-250µg/ mL	Rabbit WAP	Soler *et al*., 2005
Low-phenylalanine kappa-casein	Metabolic diseases	?	Rabbit WAP	Baranyi *et al*., 2007
Human factor VIII	Haemophilia	0.052-0.083 IU/mL	Rabbit WAP	Hiripi *et al*., 2003
		0.012-0.599 IU/mL	Mouse WAP	Chrenek *et al*., 2007

DISCUSSION

The rabbit is a standard laboratory animal that has long been used worldwide in biomedical research. Classic experimental uses of rabbits include antibody production, the development of new surgical techniques, physiology studies and toxicity tests of new drugs. Since the first transgenic rabbits were created by Hammer *et al*. (1985), many lines of transgenic rabbits have been developed and used both as animal models for a variety of human diseases and as bioreactors for the production of pharmaceutical proteins. To evaluate the current status of research in transgenic rabbits, we performed a quantitative analysis of the current publications regarding transgenic rabbits in biomedical research based on database searches.

From 1985 to 1995, the total number of publications on this subject per year reached 12, while between 7 and 20 papers were published per year from 1995 to 2008. About 74% of the publications related to transgenic rabbits originated from the USA, Japan, France, Slovakia, and Russia. The finding that the rate of publications concerning transgenic rabbits has stayed relatively stable in recent times may be because these publications primarily come from several research teams in the top five countries. It is also gratifying to see that scientists from 22 countries published papers related to transgenic rabbits. It is clear that the important role of transgenic rabbits in biomedical research has been widely accepted. While the costs of creating and maintaining transgenic rabbits are relatively higher than they are in transgenic mice, the rabbit is phylogenetically closer to primates than rodents are (Graur *et al*., 1996) and is large enough to permit non-lethal monitoring of physiological changes. A number of scientists have chosen to use transgenic rabbits as an animal model in which to study hypertrophic cardiomyopathy, lipoprotein metabolism, and atherosclerosis.

Although rodent models have provided insights into the mechanisms of cardiovascular disease, rabbits may be more suitable for the study of atherosclerosis because their lipoprotein metabolism and cardiovascular system are more similar to humans than those of mice (Fan *et al*., 2003). Because rabbit hearts are similar to human hearts but differ from those of mice, transgenic rabbits are suitable for the study of human familial hypertrophic cardiomyopathies (FHC). In mice, the most abundant component of the cardiac sarcomere, the myosin heavy chain (MyHC), consists of the ''fast'' MyHC isoform (α-MyHC), whereas the ''slow'' MyHC (β-MyHC) is the major isoform found in healthy human adults (Kavinsky *et al*., 1984). Although the rabbit atrium expresses α-MyHC at all developmental stages, the ventricles express both the α- and β-MyHC isoforms, with β-MyHC as the predominant adult isoform (James *et al*., 2000). Thus, MyHC expression in rabbits is similar to that found in the human heart. Transgenic rabbits carrying the mutant transgene β-MyHC-Q403 showed substantial myocyte disarray and a 3- fold increase in interstitial collagen expression in their myocardia (Marian *et al*., 1999). To date, transgenes for the human genes KCNQ1 and KCNH2, Myosin light chain (M149V), Cardiac troponin I (146Gly), Cardiac Gsalpha, and Phospholamban have been successfully expressed in rabbits (Pattison *et al*., 2008; James *et al*., 2002; Sanbe *et al*., 2005; Nishizawa *et al*., 2007; Brunner *et al*., 2008).

Transgenic rabbits are also widely used in lipid metabolism and atherosclerosis research. As an experimental model for the study of lipid metabolism and atherosclerosis, rabbits have several advantages over mice. These include higher levels of apoB-containing lipoproteins, abundant cholesteryl ester transfer protein, and a lipoprotein profile and a pattern of hepatic apoB100 and intestinal apoB synthesis resembling that found in humans. Rabbits are susceptible to cholesterol diet-induced atherosclerosis and form lesions resembling those seen in human atherosclerosis, with morphologies ranging from early stage lesions (fatty streaks) to complicated lesions (fibrous plaques). Transgenic rabbits have become a novel means by which to explore a number of proteins that are associated with hyperlipidaemia and atherosclerosis (Taylor & Fan, 1997; Fan *et al*., 1999b). Rabbits have been extensively utilised as an ideal model for atherosclerosis because of their size, ease of manipulation, and extraordinary response

to dietary cholesterol. Approximately 11 transgenic rabbit lines have been established for the study of atherosclerosis; the transgenes include apo(a), apoA-I, apoB, apoE2, apoE3, HL, LCAT, LPL, 15-LO, MMP-12, and APOBEC-1. These transgenic rabbits model have forged a new way to enhance our understanding of the molecular mechanisms underlying atherosclerosis.

Transgenic rabbits have also been found to be suitable bioreactors for the production of pharmaceutical proteins. Our results show that researchers and pharmaceutical companies are increasingly focusing their attention on achieving relatively large-scale production of proteins using transgenic rabbits. While mice are a good species for initially testing transgenic constructs, they are not at present useful as bioreactors for producing large quantities of recombinant proteins. The production of thousands of kg of transgenic protein demands the use of cows, while hundreds of kg can be produced in a herd of goats or sheep; however, several kg per year can be produced in rabbits. The time and expertise required to generate a transgenic founder expressing high levels of a desired protein and shepherding the purified product through clinical testing are the major drawbacks of large animal transgenic technology (Bosze *et al*., 2003). The main purpose of using bioreactors is the economical production of valuable and complex human therapeutic proteins in easily accessible fluids. The technology for using the mammary gland as a bioreactor has been developed to the point that pharmaceuticals can be derived from the milk of transgenic rabbits. The transgenic rabbit system is a lower cost alternative to larger animals, primarily because of factors such as manageable size, short lactation period, lower maintenance costs, and ability to maintain the animals under specific pathogen free (SPF) conditions. The use of transgenic rabbits as bioreactors for the production of pharmaceutical proteins is widespread in biomedical research. To date, more than 20 proteins have been expressed in the milk or plasma of rabbits. A good example is human factor IX, which is now used to treat human haemophilia B (Lubon & Palmer, 2000). Transgenic rabbits are highly suitable as an intermediate-sized animal for the production of recombinant proteins on a relatively large scale. Thus, considering both economical and hygienic aspects, rabbits are an attractive source for the mammary gland-specific expression of recombinant proteins.

Based on our findings, the most important applications in biomedical research for transgenic rabbits are as CVD models and bioreactors. Transgenic rabbits have also been used in the study of virology, immunology, diabetes mellitus, cancer, and genetics. Progress in the methods of molecular biology also promotes the development of rabbit biotechnology. Cloning of rabbits has been successfully established; this technology may lead to the establishment of knock-out or knock-in rabbits by allowing the transfer of modified nuclear material into the fertilised zygote. RNAi technology also provides another means by which gene expression could be inhibited in rabbits. It is clear that rabbits will play an increasingly important role in biomedical research as further biotechnological breakthroughs are made.

It is important to note that there are limitations to the scope of this paper. Not all biomedical journals are included in the databases we utilised; thus, we may have missed some valuable papers when we calculated the statistics for transgenic rabbit-related publications. Despite this limitation, our results are the first to quantitatively assess the developing trends regarding the use of transgenic rabbits in biomedicine.

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