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Use of Transgenic Animals to Improve Human Health and Animal Production

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Contents

Transgenic animals are more widely used for various purposes. Applications of animal transgenesis may be divided into three major categories: (i) to obtain information on gene function and regulation as well as on human diseases, (ii) to obtain high value products (recombinant pharmaceutical proteins and xeno-organs for humans) to be used for human therapy, and (iii) to improve animal products for human consumption. All these applications are directly or not related to human health. Animal transgenesis started in 1980. Important improvement of the methods has been made and are still being achieved to reduce cost as well as killing of animals and to improve the relevance of the models. This includes gene transfer and design of reliable vectors for transgene expression. This review describes the state of the art of animal transgenesis from a technical point of view. It also reports some of the applications in the medical field based on the use of transgenic animal models. The advance in the generation of pigs to be used as the source of organs for patients and in the preparation of pharmaceutical proteins from milk and other possible biological fluids from transgenic animals is described. The projects in course aiming at improving animal production by transgenesis are also depicted. Some the specific biosafety and bioethical problems raised by the different applications of transgenesis, including consumption of transgenic animal products are discussed.

Introduction

Living organisms have the capacity to evolve rapidly under the pressure of their environment. This property has been abundantly exploited by human communities since they invented agriculture and breeding. Our ancestors have thus generated the essential of the varieties and races available and used by human beings. These heavily selected species are used as a source of food as well as pets or ornamental plants. The transformations which occurred during genetic selection are so deep that a number of domesticated species would not survive without the assistance of humans. Silkworm which has become unable to move, to find its food and sexual partners is illustrating this fact.

To enhance their possible choice, experimenters currently induce numerous and random mutations in genomes using chemical compounds or irradiations. Genetics made significant progress thanks to the use of a number of drosophilae mutants. A few years ago, systematic mutations started being achieved in mice using ethylnitrosourea administered to males. In the best cases, this leads to the generation of new lines of animals showing various abnormalities mimicking more or less human diseases. This approach is not precise as a number of unknown genes are mutated with those responsible for the observed phenotypic modifications.

Conventional genetic selection does not generally imply that the involved genes are known. This method is getting improved with the increasing use of genetic markers. Yet, a new trait has limited chance to emerge in a species during an historical period of time. However, blind selection may favour the expression of genes which have deleterious side effects for consumers or environment.

Transgenesis offers quite attractive new possibilities. Indeed, it allows the stable transfer into a genome of a single known genetic information which may come from related species or not. This method is thus more precise and may generate more biodiversity than conventional selection. Yet, transgenesis may induce unpredictable side-effects due to the interference of the transgene with the host genome at its insertion site or by the interaction of the corresponding protein or RNA with cellular mechanisms.

Transgenesis has become an essential tool to study genome function. It gives to experimenters the possibility to study individual genes in their natural complex environment. Transgenesis may contribute to create new relevant models for the study of human diseases. Transgenesis may also contribute to reduce rejection of some pig organs to be grafted to humans. Some pharmaceutical proteins are being prepared from the milk of transgenic animals. Moreover, animal productions may be improved by transgenesis.

Despite numerous successes, transgenesis use is still limited by technical problems which are progressively solved. Gene transfer remains poorly efficient in some species and transgene expression and interference with the host genome are not fully controlled. The relevance of the transgenic animals used as models or as a source of products for humans remains dependent on technical progress.

Techniques of Transgenesis

Transgenesis is facing different problems: gene availability, gene transfer, construction of vectors allowing reliable transgene expression, and interpretation of the data. The complete genome sequencing in several animal species including man (International Human Genome Sequencing Consortium, IHGS 2004), mouse and rat is already providing experimenters and biotechnologists with a large number of genes. The genome sequencing in several farm animals like cow, chicken (Schmutz and Grimwood 2004), rabbit and salmon which is in course will still add genes to the list. Interestingly, a project which started recently aims at sequencing the whole genome of 15 mouse strains. These strains have been chosen among those which show the largest biological

diversity. Correlations between phenotypic properties of animals and gene sequences should offer to researchers the possibility to evaluate allele effects in transgenic animals (Pearson 2004).

The interpretation of data obtained with transgenic animals will always remain difficult in a number of cases. This is the price to pay as soon as genes come back to whole living organisms. Progress is being made to generate transgenic animals and to express transgene in an appropriate manner. Additional improvement of the available techniques are still needed.

Gene Addition and Replacement

In most cases, foreign DNA is integrated randomly in host genome or at least in an uncontrolled manner. When linear DNA is introduced directly into the nucleus (thus via microinjection) it is circularized, randomly cleaved and multimerized according to a homologous recombination process generating well-shaped tandem concatemers (Houdebine 2003). When linear DNA is introduced in the cytoplasm, either by microinjection, transfection, electroporation, etc. it forms head to tail and tandem concatemers according to a random process. This event is often accompanied by DNA rearrangement. Moreover, transgenes integrated as head to tail polymers are often poorly expressed. At a low frequency, foreign DNA sequences may recombine with homologous host sequences leading to precise gene replacement.

Nake DNA microinjection

To generate lines of transgenic animals harbouring the foreign gene in all cells, the DNA must be present in the embryo at the one cell stage. The most commonly used method consists of injecting linear DNA into a pronucleus of one cell embryos. This technique was successful for the first time in 1980 and made little progress thereafter. Up to 1–3% of mouse microinjected embryos commonly become transgenic animals. This yield can be significantly enhanced by using mouse strains like FVB/N (Auerbach et al. 2003). For unknown reasons, this yield is lower or much lower in rabbits, rats, pigs and ruminants respectively. This is not because of microinjection *per se* but to the integration process. Thus gene addition to ruminants is now achieved using essentially the cloning approach (see below).

Pronuclei are visible only in mammals. In lower vertebrates and invertebrates, DNA can be injected only in embryo cytoplasm. About 1000–5000 copies are injected into pronuclei and up to 1–20 million copies must be injected in cytoplasm of other species. For unknown reasons, nake DNA does not integrate at a significant rate in the genome of animals like chicken, xenopus and the fish medaka. Interestingly, electroporation of medaka embryo can generate a high number of transgenes (Hostetler et al. 2003). The integration yield may be enhanced by adding the recombinase Rec A to the DNA (Maga et al. 2003). More impressive is the fact that fragments of interest released from plasmids inside the embryo by the meganuclease I-Sce I show a high capacity to integrate into medaka genome (Thermes et al. 2002). The same phenomenon was observed in xenopus. The mechanism of action I-Sce I is not fully understood. It has not been proved yet that a significant increase of transgenesis yield can be obtained in mice and other mammals using this technique.

Use of transposons

Transposons are genomic DNA sequences capable of autoreplicating and integrating randomly in additional genome sites. This property is being used to transfer foreign genes into genomes. Transposon P is extensively used to generate transgenic drosophilae. A few transposons proved efficient to transfer foreign genes in species in which nake DNA does not integrate. Transposons have thus been designed to generate transgenic medaka (Dupuy et al. 2002), silkworm (Tamura et al. 1999) and a number of invertebrates. The method using transposons is getting improved (Mikkelsen et al. 2003; Masuda et al. 2004). Transposons are efficient and reliable tools to generate transgenic animals but they cannot harbour more than 2–3 kb of foreign DNA.

Use of lentiviral vectors

Retroviral vectors have been extensively studied for human gene therapy and in some cases used successfully (Chan et al. 2001). A quite significant improvement has been achieved with the use of lentiviral vectors. These vectors have the capacity to cross the nuclear membrane and reach host genome in cells at any phase of their cycle, including in quiescent and embryonic cells. Moreover, the envelope from Vesicular Somatitis Virus (VSV) may be added to the lentiviral particles. This allows a concentration of the particles by ultracentrifugation and a high rate of infection. In addition, VSV envelope recognizes no particular receptors but membrane phospholipids. This property allows the infection of essentially all cell types (Lois et al. 2002).

This tool facilitates the generation of transgenic mice as the viral particles harbouring the foreign DNA must be injected between zona pellucida and the embryo membrane rather than in pronuclei as it is the case for nake DNA. The use of lentiviral vectors has been extended successfully to other species (Fassler 2004; Pfeifer et al. 2004; Whitelaw 2004), such as chicken (McGrew et al. 2004), cow (Hofmann et al. 2004), and pig (Hofmann et al. 2003; Whitelaw et al. 2004).

For unknown reasons, lentiviral vectors have to be injected in early pig embryos but in cow oocytes to generate the higher number of transgenic animals. Experiments carried out with monkey embryos indicated that lentiviral vectors did not allow the generation of transgenics (Wolfgang et al. 2001). In farm animals, the overall efficiency of transgenesis appears up to 50-fold with lentiviral vectors than with DNA microinjected into pronuclei. Conventional retroviral vectors carrying VSV envelope (Koo et al. 2004) or avian viral envelope (Ivarie 2003) was also efficient to generate transgenic chicken.

Commercial kits are available to generate efficient and safe lentiviral particles to generate transgenic animals. Lentiviral vectors generally allow a reliable expression of transgenes. Their major limitation is that they cannot

harbour more than 8–9 kb of foreign DNA and they integrate preferentially within coding region of host genes. It is also sometimes difficult to use specific promoters to drive the expression of the transgene as the Long Terminal Repeat (LTR) of the vector may interfere with these promoters.

Use of episomal vectors

An alternative to integration consists of using gene constructions capable of autoreplicating and being efficiently dispatched in daughter cells. Plasmids containing a MAR sequence (matrix attached region) can be stably maintained in cell lines (Lipps et al. 2003). It is not known whether these vectors can generate transgenic animals. Fragments of chromosomes can also be used to transfer foreign genes into cells (Lindenbaum et al. 2004) or transgenic animals (Kuroiwa et al. 2002).

Use of gametes

Experiments described above indicate that in some species, namely in cow, gene transfer into embryos using lentiviral vectors is efficient. Gene transfer into gametes is a possible alternative. Experiments carried out more than one decade ago showed that mouse sperm incubated with DNA and further used for fertilization can generate transgenic animals. This method known as spermatotransgenesis is not easily reproducible due to the presence of DNAse I on the surface of sperm. Yet, it has been confirmed in pig (Lavitrano et al. 2002, 2003) and also in sheep. It also allowed gene transfer into rabbit oocytes (Wang et al. 2001, 2003).

A degradation of sperm membrane followed by an incubation with DNA and fertilization using intracytoplasmic sperm injection (ICSI) proved efficient in xenopus (Marsh-Armstrong et al. 1999) and also in mice (Kato et al. 2004; Moreira et al. 2004). Interestingly, DNA fragments as long as 200 kb incubated with mouse sperm and used for ICSI could generate transgenic animals at an acceptable rate (Moreira et al. 2004). ICSI appeared unable to generate transgenic rhesus monkey (Chan et al. 2000). It is not clear whether, in these experiments, sperm membrane was degraded before incubation with DNA.

The yield of spermatotransgenesis has been improved by using a monoclonal antibody which binds specifically sperm cell surface by recognizing a specific antigen and DNA by its C-terminal end. This complex proved quite efficient to generate several transgenic species (Chang et al. 2002; Wang 2003). Foreign DNA can also be transferred directly into sperm precursors by injecting DNA-transfectant complex into seminal tubules (Celebi et al. 2002). Alternatively, sperm precursors may be collected, transfected *in vitro* and reimplanted into recipient testis (Honaramooz et al. 2003; Readhead et al. 2003; Oatley et al. 2004). This approach could allow gene addition and replacement.

Use of pluripotent cells

Embryonic stem cells (ES cells) can be cultured and use to replace host genes by homologous recombination (Capecchi 1989). This allowed specific knock out of about 5000 genes in mice (see below). For unknown reasons, this method can be implemented with ES cells from only two mouse lines. This seems due to the loss of cell pluripotentcy during culture. The cells transplanted into recipient blastocysts generate then, at best, chimeric animals but with no transmission of the mutation to progeny. Experiments in course aim at identifying the key genes involved in the maintenance of pluripotency and using them to establish functional ES cells in various mouse strains as well and in different species.

Use of somatic cells and nuclear transfer

Soon after the birth of Dolly, it was shown that DNA transfer into somatic cells further used to generate cloned transgenic animals, although laborious, was more efficient than classical microinjection to obtain transgenic ruminants (Schnieke et al. 1997; Cibelli et al. 1998). Gene replacement in sheep was achieved 1 year later using the same technique (McCreath et al. 2000) and later in pig (Dai et al. 2002; Lai et al. 2002). Quite interestingly, a recent publication indicates that the two alleles of the PrP gene involved in prion diseases and the two alleles of immunoglobulin μ gene have been knocked out in the same cow (Kuroiwa et al. 2004). To minimize side-effects of cloning, the foetus obtained after the first allele replacement was the source of cells for a second allele replacement followed by a second cloning. This protocol was repeated for the two alleles of the second gene.

Targeted Gene Integration

Homologous recombination between a genomic sequence and a foreign DNA fragment theoretically allows replacement of any region of a genome. The most frequent use of this technique known as knock out consists of replacing an active gene by an inactive version of the gene. A genomic active gene may as well be replaced by another active gene related or not to the targeted gene. This approach known as knock in allows the evaluation of the biological activity of different alleles in their natural position in genome.

Knock in may also be implemented to integrate a foreign gene in a given genomic site. This site may have been chosen because it contains regulatory sequences allowing reliable expression of the integrated foreign gene. The DHFR gene locus is thus used to express transgenes. The DHFR gene is expressed in all cell types and it was originally used for expression of naturally ubiquitously expressed transgenes (Bronson et al. 1996).

More recent studies revealed that this genomic site favours expression of multiple foreign genes in a cell-specific manner as soon as an appropriate promoter is associated to the transgene (Farhadi et al. 2003). This approach is now extensively used by a private company which generates successfully transgenic mice in which the transgene is appropriately expressed. This method could be extended to other genomic sites but also to other species. The limitation of this approach remains the capacity to generate living organisms from the cells in which the foreign gene has been added.

The rate of homologous recombination is low in vertebrates and particularly in somatic cells. Engineered genome may facilitate gene targeting. One possibility consists of introducing the site of a meganuclease such as I-Sce I in a given region of a genome. The site may have been chosen before introducing the meganuclease site. The introduction of this site must then be achieved using first a homologous recombination. Alternatively, the I-Sce I site may have been introduced randomly in the genome and only the sites showing expected properties are retained. This meganuclease site and others have been retained for two reasons: (i) they are quite rare and have a negligible chance to be naturally present in a vertebrate genome, and (ii) they allow the clivage of both DNA strands after addition of the enzyme. This cleavage stimulates strongly the repair mechanism and favours greatly the targeted integration of a foreign DNA at this site according to a homologous recombination process (Cohen-Tannoudji et al. 1998).

In future, the technique might be applied to pre-existing genomic sites not corresponding to native meganucleases. Indeed, existing meganucleases may be engineered to recognize specifically chosen natural sites in a genome (Epinat et al. 2003). This ambitious project aims at correcting mutated human genes such as those coding for coagulation blood factors. This method might be used as well to generate new alleles in farm animals.

Other specific recombination sites may also be initially introduced in a genome to target the integration of foreign DNA. Two systems are currently used. One known as Cre-LoxP comes from a bacterial phage and the second Flp-FRT comes from yeast. The short DNA sequences LoxP or FRT may be first introduced in genomes as depicted above. The addition of LoxP or FRT sequences at the end of foreign DNA allow their specific integration at their corresponding sites in the genome as soon as the recombinases Cre or Flp are added to cells respectively.

Improvement of the method has been achieved by adding LoxP or FRT sequences at both ends of the foreign DNA fragments. The details of this method known as recombinase-mediated cassette exchange have been described in several reviews (Bode et al. 2000; Baer and Bode 2001).

The possibility to introduce different genes or gene versions in the same site of a genome offers the advantage of reducing the side-effects of random integration. The position effects of chromatin environment on transgenes may still exist with the targeted integration but they are always the same. This may simplify greatly interpretation of the data obtained with transgenic animals to study gene function or human diseases.

In the majority of the cases, gene knock out by homologous recombination is achieved in ES cells further used to generate chimeric transgenic animals as well as in somatic cells used to generate transgenic cloned animals. In these two situations, the targeted genomic gene is inactivated in the very early stage of embryo development. For a number of reasons, it may be preferable to delay gene knock out. This may be achieved by introducing two LoxP sites within the gene to inactivate, using conventional homologous recombination. The addition of Cre recombinase triggers elimination of the sequence located between the two LoxP sites leading to an inactivation of the targeted gene. Cre recombinase gene may be expressed only in a given tissue of the animal harbouring the gene under the control of a cell-specific promoter. Alternatively, Cre gene may be brought to a tissue of adults using adenoviral vectors.

Design of Vectors for Transgene Expression

Transcribed and not transcribed regions of genes contain multiple signals for transcription, mRNA maturation and transfer to cytoplasm, mRNA stability and translation. Gene construction is empirical and it often leads to suppression or addition of unknown signals. This gives limited chance for the transgene to be expressed efficiently.

Vectors for a reliable transgene expression

A certain number of rules have been defined to tentatively optimize transgene expression (Houdebine et al. 2002a). Transgenes must contain at least one intron which favours premRNA maturation and mature mRNA transfer to cytoplasm. Transgenes must not contain too many GC rich regions and particularly CpG motifs in their promoters. These structures are recognized as foreign elements by the cells, probably during early embryo development, and their C becomes methylated. This inactivates promoters in a manner which is reversible or not and which may be transmitted to progeny. Transgenes must be preferably integrated in a low copy number. This cannot be controlled in practice unless the gene construct contains a LoxP sites which allow elimination of transgene copies but one under the action of Cre recombinase.

The first experiments performed in the early 1980s revealed that the transgenes are often poorly expressed and under the partial control of host enhancers present in the vicinity of their integration sites. Gene studies performed essentially in human, as well as transgenesis carried out in drosophilae and mice indicated that remote sequences are required for genes to be expressed in an appropriate manner. These remote regions are known as locus control region or insulators. The study of these regulatory regions is underway in a few systems. It appears now that insulators contains several kind of elements: (i) potent enhancers which are often cell specific, (ii) enhancer blockers which prevent cross talk between neighbour genes in a genome, and (iii) chromatin openers which induce local post-translational histone modifications which allow the transcription machinery to reach the genes to be expressed.

Different elements of a limited number of insulators have been identified. They may be located in a single region forming a barrier insulating a locus from its neighbours (Bell et al. 2001). It seems however that in most, if not all cases, quite distant elements participate to the insulator effects. The regulatory elements of a gene may be separated from its promoter by one or several non-related genes. Thanks to a looping process, the distant regulatory elements associated to

the corresponding transcription factors move to the promoter region forming a hub which induces histone hyperacetylation and a local opening of chromatin. This allows the transcription machinery to express the gene (De Laat and Grosveld 2003). These dynamic modifications of chromatin conformation more and more appear not only as mechanisms controlling gene expression but also as mechanisms of differentiation. The study of albumin gene illustrates this point. This gene is in closed chromatin conformation in early embryo. It becomes open specifically in endoderm and remains so until hepatocyte differentiation. It remains closed in the other differentiated cells (Lomvardas and Thanos 2002).

These phenomena were discovered and are still studied thanks to the use of transgenesis. Transgenesis is itself highly beneficial of these discoveries. Indeed, the addition of insulator elements, namely of the 5′HS4 region from the chicken β-globin locus, greatly favours expression of transgenes in vertebrates (Giraldo et al. 2003).

Long genomic DNA fragments (100 kb or more) have much chance to contain insulators which allow an appropriate expression of the transgenes present in the fragment (i.e. expression in all transgenic lines, tissue-specific expression, expression as a function of the integrated copy number). This phenomenon was observed in about 20–30 cases, including for the pig milk protein gene WAP (whey acidic protein) (Rival-Gervier et al. 2002). This suggests that in a few years, genes of interest will be introduced into long genomic DNA fragments to optimize their expression as transgenes. An increasing number of validated genomic fragments will become progressively available allowing satisfactory transgene expression in most cell types. It is also conceivable that the naturally remote regulatory elements of a locus will be concentrated into a compact insulator more easily manipulated.

Vectors for the specific inhibition of host gene expression

The use of animal models to study gene function as well as human diseases, and also a number of biotechnological applications imply the inhibition of host genes (including viral genes). This goal may be reached by different methods. Gene knock out described above is one of these methods. This quite potent approach clearly suffers from a lack of flexibility. Indeed, ideally, a host gene should be inhibited and reactivated at any period of life and in any tissue. The inhibition of a gene may take place at different levels: the gene itself, the mRNA or the protein. Existing tools to inhibit gene expression at these different steps are being improved.

Use of RNAi

The addition of complementary synthetic oligodeoxyribonucleotides to cells *in vitro* or *in vivo* may inactivate the corresponding mRNA but in a local and transient manner. Expression of antisense single-strand RNAs having or not a ribozyme activity in transgenic animals proved efficient in some cases to inhibit endogenous genes. This approach is in practice and not extensively used as it remains difficult to design antisense RNAs capable of interacting efficiently with their targets.

A fortuitous discovery in 1988 revealed that, unexpectedly, long double-strand RNAs have much more potent capacity to destroy the corresponding mRNAs than single-strand complementary RNAs (Fire et al. 1998). The mechanisms implied in this phenomenon have been essentially deciphered. Long double-strand RNA are cleaved by an enzymatic complex (DICER) into 21–23 bp fragments known as siRNA (small interfering RNA). Each fragment is associated with a protein complex (RISC) which cleaves quite specifically, the corresponding monostrand RNA in cytoplasm (Novina and Sharp 2004).

These observations match with the well-known post-transcriptional gene silencing (PTGS) which is frequently observed in transgenic plants. Transgenes are frequently silent in plant. More unexpectedly, endogenous genes having sequences similar to some of the transgenes may also become progressively silent. This phenomenon was not reported in transgenic animals. This may be due to the fact that in plants, transgenes are frequently integrated in head to tail concatemers generating siRNAi. This is also because of autoamplification of RNAi which occurs in plants and lower invertebrates but not in vertebrates (Novina and Sharp 2004).

Long double-strand RNAs induce interferon and cell death in vertebrates. The RNAi effect can therefore be obtained only by using synthetic 21–23 bp RNA or vectors generating directly a functional RNAi. Vectors containing RNA polymerase III promoters can efficiently express RNAi in stable cell clones but unfortunately not in transgenic animals unless they are introduced in retroviral vectors (Unwalla et al. 2004). Alternatively, vectors containing minimum promoter and terminator could be used. An elegant work indicated that a vector designed to express in cytoplasm generated siRNA in the nucleus and induced a specific RNA interference. The mice obtained by this method did not express ski gene and were phenotypically similar to those in which ski gene was knocked out (Shinagawa and Ishii 2003). This approach has not yet been extended successfully to other genes.

Independent observations have shown that RNAi are more or less potent according the targeted mRNA sequence. The most efficient RNAi can be found empirically using RNAi libraries (Sen et al. 2004; Shirane et al. 2004). Consensus sequences has been found in efficient RNAi and their activity can now be predicted to a large extent (Hohjoh 2004; Mittal 2004; Reynolds et al. 2004; Ui-Tei et al. 2004; Yoshinari et al. 2004; Williams 2005).

Plant and animal genomes contains at least 100 genes coding for short hairpin RNAs known as microRNAs (miRNAs). The miRNAs are transcribed as precursors (primiRNAs) containing about 120 nucleotides. They are processed by enzymatic complexes. The mature miRNAs show high homologies with siRNAs may be partially complementary to the 3′ untranslated region (3′UTR) of an mRNA. In these conditions, they inhibit mRNA translation. The miRNAs may be strictly complementary to an mRNA. They then act as siRNAs and degrade the targeted mRNA. The miRNA genes are transcribed by RNA polymerase II. Vectors may be designed to direct the expression of small RNAs acting

as siRNAs or miRNAs to inactivate specifically targeted mRNAs (Novina and Sharp 2004).

A recent study showed that the degradation of introns generates small RNA fragments which are processed and recruited to act as siRNAs or miRNAs (Ying and Lin 2005). This natural process may play an important role in the regulation of gene expression. This important discovery offers additional possibilities to use introns as a source of small RNAs capable of inhibiting specifically gene expression in transgenic animals. To reach this goal, sequences complementary to the targeted mRNA have to be added within introns.

The PTGS has been observed in all eucaryotes so far. Another phenomenon, TGS (transcriptional gene silencing), was described years ago in transgenic plants but not in animals. The TGS mechanism is now partially understood. siRNAs recognized genome sequences and induce local cytosine methylation and particularly in CpG motifs frequently involved in promoter activity. Methylation of active promoter regions silences the corresponding gene and this inactivation is transmitted to daughter cells and progeny. Quite interestingly, TGS was recently shown to occur in mammalian cells (Kawasaki and Taira 2004; Morris et al. 2004).

Experimenters have now unexpected and beyond hope new tools to specifically and reversibly inactivate endogenous genes. This process is known as knock down. These tools are already being extensively used in C. Elegans, a nematode, in which 5000 genes have been knocked down generating 1500 phenotypic traits. The same appears possible for vertebrates. Undoubtedly, this is expected to have a strong impact on human health. Indeed, siRNAs and miRNAs synthesized chemically or by vectors transferred to cells may inhibit viral genes or endogenous genes involved in cancer development (see a multiarticle review in Nature (2004) 431: 337–378). Experimenters may take advantage of the numerous companies involved in RNAi use (Clayton 2004).

Use of transdominant negative proteins

The action of a gene can be blocked at the protein level by expressing specific inhibitors such as antibodies recognizing the protein of interest (Müller 2000). Alternatively, transdominant negative proteins acting as decoys may be used. Transgenic mice mimicking type II diabetes were obtained by overexpressing a mutated insulin receptor still capable of binding the hormone but not of transducing its message (Chang et al. 1994). Similarly, overexpression of pseudorabies virus receptor in transgenic mice protect these animals against Aujeszky disease (Ono et al. 2004).

Genetic ablation

Destroying specifically given cell types in animals may reveal their role in organogenesis. This can be achieved by expressing genes coding for toxins. The challenge is then to express quite specifically the transgenes. Different systems are implemented for this purpose (Saito et al. 2001; Chen et al. 2004). They rely on two-step mechanisms which reduce the risk of ectopic expression of the toxin genes.

Vectors for the Conditional Expression of Transgenes

In a number of situations, it is highly desirable to control the expression of a transgene by inducers not acting on endogenous genes. This can be achieved by several systems all based on the use of transcription factors engineered to be sensitive to molecules such as antibiotics. These systems have been described in recent reviews (Houdebine 2003; Weber and Fussenegger 2004). A limitation of these systems is the too high background expression of the transgenes in the absence of inducers. Several improved systems have been proposed. They imply the action of transcription factors controlled by the inducers and acting alternatively as enhancers or silencers (Jiang et al. 2001; Weber and Fussenegger 2004). Transgene expression can also be controlled at the translation level (Boutonnet et al. 2004).

Applications of Transgenesis

Study of gene function and human diseases

The major use of genetically modified organisms is to get knowledge on gene function and regulation, as well as on human diseases. The tools depicted above have been greatly improved and they contribute to reach this goal. Up to 300 000 lines of transgenic mice are expected to be generated in the two coming decades. The 20 000–25 000 genes of mice will be knocked out or down. The ES cell lines in which essentially all the mouse genes are knocked out and thus ready to generate mutated animals should be available in no more than 5 years (Abbott 2004). The list of knock out mice may be consulted at the following address: http://research.bmn.com/mkmdj.

Homologous recombination in bacteria using long DNA fragments in BAC vectors (bacterial artificial chromosomes) facilitate the preparation of numerous mutated ES cells and the generation of a number of knock out mice (Valenzuela et al. 2003). Transgenic animals are used to study human diseases as models in quite different fields: genetic diseases, infectious diseases, neurodegenerative diseases, cell apoptosis, ageing, arteriosclerosis, cancer, xenografting, endocrinology, metabolism reproduction and development (Houdebine 2004).

More and more sophisticated models are being prepared to mimic the human diseases as much as possible. Mouse lines harbouring different alleles of the same human gene are thus prepared by knock in to evaluate their involvement in the efficiency of new pharmaceutical molecules. This reduces the number of phase III assays to be performed in humans (Liggett 2004). In some cases, the Cre recombinase used to trigger a conditional gene knock out is more precisely expressed when its gene is inserted into a long genomic DNA fragment in BAC vectors. A number of gene knock out are lethal in the early stage of embryo development. This is the case for Rb gene. Chimeric embryos formed by tetraploid Rb+/+ placenta cells and Rb−/− inner cell mass can develop and allow a study of Rb gene inactivation in adult.

Gene knock out may be lethal because one essential organ has become no more functional whereas other interesting effects are supposed to take place in other organs. In these cases, the function of the organ responsible for animal death may be restored by expressing specifically the normal gene in this organ of transgenic mice. The effects of the knock out gene can then be studied in the other organs of the animals (Lee and Threadgill 2004).

Mouse in a good model to study many but not all the human diseases. Indeed, mice, as humans, are mammals among others. Some functions are too different in the two species. This is the case for lipid metabolism and arteriosclerosis. For this reason, transgenic rabbits are extensively used to study human diseases resulting from disorders of lipid metabolism (Fan and Watanabe 2003). Pigs could be used for the same kind of study but transgenesis is more complicated and costly in this species than in the rabbit. Other diseases can successfully be studied using transgenic pigs as models (Kues and Niemann 2004). Rat is more appropriate for some specific diseases. It is important to note that the recent success for cloning rat allow theoretically gene knock out in this species and thus the creation of new relevant models (Zhou et al. 2003).

Other specific cases are also worth being considered. Homozygous goat lines without horns are subfertile. It has been shown that this is due to a mutation responsible for an abnormal differentiation of foetal gonads (Pailhoux et al. 2001). This abnormality is similar to some human genetic diseases. Mice are not relevant models for this study (Vaiman 2003). Transgenic goats obtained by cloning are currently being used for this purpose (E. Pailhoux, personal communication).

GFP gene (green fluorescent protein) has been transferred to most of the species in which transgenesis is possible. Rabbits expressing GFP in all cell types (Boulanger et al. 2002) are being used to follow cell fate in chimeric embryos as well as in normal rabbits in which organs have been grafted. Rabbits in this case are preferred as having organs larger and more easily transplanted than mice.

Adaptation of pig organs for transplantation to humans

The idea of transplanting animal organs to humans is not new. It was tested for the first time one century ago. The transplantation was a success in a number of cases from a surgical point of view. The grafted organs generally did not survive as they were strongly rejected. This approach was abandoned until the discovery of immunosuppressors currently used for allografting. The use of immunosuppressors did not reduce rejection of xeno-organs. This indicated that at least some of the mechanisms involved in xeno-organ rejection are different of those which operate after allotransplantations.

A systematic study of xeno-organ rejection revealed that at least three mechanisms are involved. The first known as hyperacute rejection operates as soon as the xeno-organ is in contact with the blood of the recipient. It was shown that natural antibodies already present in recipient blood before transplantation recognize antigens located at the surface of the foreign cells, particularly of the endothelial cells of the xeno-organs. This induces a quick activation of host complement which destroys the endothelial cells and provokes thrombosis followed by organ death. The major pig antigen has been identified. It is composed of carbohydrate moieties ended by the 1,3-α-galactose-1, 3-α-galactose motif covalently added to a number protein present at the surface of the cells. The primates of the Old World including human have no more a functional α-galactosyl transferase gene which is responsible for the addition of α-galactose to proteins. Higher primates have natural antibodies against the 1,3-α-galactose motif. The presence of these antibodies does not result from an immunoreaction induced by the presence of the foreign cells. This is the reason why the first rejection mechanism is so fast and potent. It was shown that a removal of these antibodies from recipient blood prevented xeno-organ rejection but only transiently until the reappearance of the antibodies.

Transgenesis appeared not only a mandatory tool to study rejection mechanisms but also potentially to engineer pigs to be used as organ donors for humans. Experimental study of rejection mechanisms is being carried out in mice, rats, rabbits and more rarely directly in pigs. Mice are too small to allow easy organ grafting to experimental recipient primates. Rats and rabbits are more appropriate (Houdebine and Weill 1999).

Mice, rats, rabbits and pigs expressing the natural antihuman complement genes, DAF, CD59 and a few other genes have been obtained by different laboratories and companies. Kidneys from these transgenic pigs have been maintained for 2–12 weeks in monkeys treated by conventional immunosuppressors whereas control pig kidneys were destroyed after one or a few days. This pioneer work is a proof of concept that transgenic pigs could be the source of organs for humans on condition to know which genes should be added to or deleted from the pig genome.

These results have been elegantly confirmed by two independent studies. The 1,3 α-galactosyl transferase gene has been knocked out using homologous recombination and cloning techniques. The kidneys from these animals showed no more hyperacute rejection when grafted to immunosuppressed monkeys (Dai et al. 2002; Lai et al. 2002). Other genes involved in the second and third rejection mechanisms, acute vascular and cellular rejection respectively, are under study using the same technical approach.

Pig has been chosen as a potential donor of organs and cells for humans for several reasons. Pigs and humans have organs of similar size and a metabolism showing many similarities. Pig can be bred in pathogen-free conditions. Transgenesis is possible in this species and pig is also neither too close nor too far from human. Primates would probably be better donors but these animals have higher chance to transmit infectious diseases to recipients. These animals are also protected for ethical reasons and their production cost is particularly high.

A few years ago, it was observed that a non-pathogen retrovirus PERV can be transmitted to some human cell lines maintained in culture. A close examination of this phenomenon showed that this virus has very little

chance to be transmitted to humans (Switzer et al. 2001). It remains that the transmission of infectious pathogens from pig organs or cells to patients cannot be excluded. This possibility is enhanced by the fact that the pig organs would be engineered to be less sensitive to the immunological defence of the recipients who, in addition, would be treated by immunosuppressors. These problems do not appear insurmountable.

Interestingly, pig strains expressing no retroviral sequences have been found (Oldmixon et al. 2002). It is also conceivable that transgenes could be used to prevent replication of pathogens in pigs. Patients might also be vaccinated against some of the pig pathogens. Although a number of hurdles remain, it appears possible that before the end of this decade, a few hearts, kidneys, neurones and perhaps lungs and pancreas from transgenic pigs will be transplanted to a few patients.

Production of pharmaceutical proteins by transgenic animals

The use of proteins as therapeutics is quite logical, yet it became a reality only in the first part of the last century. Our ancestors who used essentially plant extracts absorbed orally as pharmaceuticals could not imagine that proteins could play an important role to treat patients. The market of therapeutical proteins is presently in full expansion (Pavlou and Reichert 2004). Some experts are convinced that the need of pharmaceutical proteins is increasing so much in the coming decade that all the available production systems might not be sufficient to meet the demand.

Proteins can be extracted from human blood or organs but this method may be either insufficient or risky for patients and not always ethically acceptable. In the early 1980s, human proteins started being produced by recombinant bacteria. The idea of using transgenic animals as bioreactors emerged in 1982 when it was shown that the giant transgenic mice had up to microgram quantities of human growth hormone in their blood.

In 1986, it appeared that milk should be the best animal system to produce pharmaceutical proteins at an industrial scale. The proof was experimentally given the following year when transgenic mice produced active human TPA and ovine-β-lactoglobulin in their milk. This production system is presently the most mature and the first protein, human antithrombin III is under evaluation by the European Medicament Evaluation Agency. Quite different proteins are being produced: blood factors, albumin, enzymes, spider silk, vaccines and mainly monoclonal antibodies.

About 100 of proteins have been experimentally produced in milk and five to 10 of them are under clinical studies. Among the proteins which could be put in the market in the coming years are human antithrombin III (Meade 1999), a vaccine against malaria (Stowers et al. 2002), human C1 inhibitor (Koles et al. 2004) and a vaccine against rotavirus (Soler et al. 2004).

It is now admitted that milk may be a major source of pharmaceutical proteins. This system can produce very large amount of proteins at a low cost. Post-translational modifications are achieved by the mammary cells. Yet, some proteins are produced only at a low level. They cannot be completely glycosylated or cleaved and they may exert some deleterious effects on the animals.

Animals can be engineered to improve post-translational modifications of recombinant proteins. This was achieved with transgenic mice overexpressing furin gene in their mammary gland. This allowed recombinant protein C to be more completely cleaved and activated (Drews et al. 1995). Genes coding for glycosylating or γ-carboxylating enzymes might be added as well to animals.

Production of pharmaceutical proteins in milk raises little ethical and no environmental problems. Several species, rabbits, sheep, goats, pigs and cows are being used to produce pharmaceutical proteins. Each species has advantages and drawbacks according to the quantity of proteins to be produced and their required post-translational modifications. Purification of recombinant from milk may be uneasy in some cases due to the huge amount of milk proteins. Other systems such as egg white, seminal plasma, silkworm sericigene gland could be used in future with no evidence that they have clear advantages over milk (Lubon 1998; Houdebine 2000, 2002a; Nikolov and Woodard 2004). The possible transmission of pathogens present in milk to patients does not appear a crucial problem although it raises concerns. This is the case for prions. Interestingly, rabbit is a species insensitive to prions.

Other systems can compete with animal bioreactors. Bacteria are often appropriate when no post-translational modifications of the proteins are needed. Yeasts do not glycosylate proteins as animal cells do. Yet, recent work showed that engineered yeast can add complex carbohydrate motifs to proteins (Hamilton et al. 2003). Plants can produce quite large amounts of pharmaceutical proteins at a low cost. The proteins are in this case correctly processed but not perfectly glycosylated. Engineered plants can add sialic acid to the proteins. Plants have no significant chance to transmit pathogens to humans. It remains to find systems preventing all dissemination of recombinant proteins from plants cultured in open fields (Horn et al. 2004).

Improvement of animal production

Transgenesis is expected to improve animal production as conventional selection did and is still doing (Houdebine 2002b; Houdebine et al. 2002b; Clark and Whitelaw 2003; Niemann and Kues 2003; Zbikowska 2003). United Nations recommended the implementation of the transgenic approach to improve health in developing countries, even if this recommendation concerns presently mainly crops (Acharya et al. 2003). The major domains in which transgenesis is expected to have an impact are the followings: health, growth, milk and carcass composition, wool growth and composition, as well as environment.

The struggle against animal diseases appears presently the most important issue. Indeed, this would (i) reduce the use of drugs and particularly of antibiotics in some cases, (ii) enhance animal welfare, (iii) facilitate breeder task, (iv) reduce loss and enhance yield in breedings, and (v) reduce the frequency of animal disease transmission

to humans. Some of the techniques described above are appropriate to reach this goal.

Examples may illustrate the trends in this field. Mice and expectedly soon pigs expressing a soluble form of pseudorabies virus are protected against Aujeszky disease (Ono et al. 2004). Cows in which the PrP genes has been inactivated by homologous recombination are expected to be insensitive to prion diseases (Kuroiwa et al. 2004).

Milk of some transgenic animals contains proteins having antibacterial activities: human lactoferrin (Zuelke 1998), lysostaphin (Mitra et al. 2003) and human lysozyme (Murray et al. 2003). These proteins are expected to protect both consumers and mammary gland of animals against bacterial infections. Mouse milk containing a recombinant antibody against coronavirus can protect pups against infection by the virus (Castilla et al. 1998).

Rotavirus antigens VP_2 and VP_6 have been produced in rabbit milk. They protect partially or completely adult mice against the virus after a vaccination using different administration routes. Extracts from this milk might be used to vaccinate children and animals at a large scale (Soler et al. 2004).

Transgene mice expressing a lactase gene in their milk have reduced content of lactose to which a large proportion of humans is intolerant (Jost et al. 1999). Transgenic pigs secreting in their milk bovine α-lactalbumin or IGF1 have a higher capacity to feed their pups (Bleck et al. 1998). Transgenic cows overexpressing cow β- and K-casein genes have been obtained and are under study (Brophy et al. 2003). Attempts to improve wool composition met disappointing success so far (Bawden et al. 1999).

Pig expressing *Escherichia coli* phytase gene in their saliva excrete 75% less mineral phosphate leading to a significant reduction of pollution (Golovan et al. 2001). Transgenics pig expressing $\Delta 12$ fatty acid desaturase gene from spinach contain more linoleic acid in their adipocytes (Saeki et al. 2004).

The most advanced project in this field is certainly the fast-growing fish harbouring additional copies of growth hormone genes. Several species, salmon, trout, tilapia, carp, loach, cat, fish and several others are currently under study. Nothing seems to indicate that these products would raise problems for human consumers. It is therefore possible that the authorization to put this transgenic fish in the market will be given in the coming years (Muir 2004).

Conclusion and Perspectives

The impact of animal transgenesis on human health is still limited but quite significant. Progress is being made in the different domains. Recent technical advances, namely gene addition and replacement using cloning, have opened new avenues. The complete genome sequencing of several species of farm animals is providing researchers with additional genes of interest. This will also enhance the chance to generate beneficial new lines of animals.

The use of transgenic animals to study human diseases raises no particular problems but ethical. The same is true for the animals used as a source of organs or pharmaceutical proteins. The medical problems raised by the use of pharmaceuticals proteins are under the evaluation of commissions which have a long experience in this field with the conventional chemical drugs. The implementation of transgenesis to improve animal production appears more complex.

Despite recent progress of gene transfer techniques, the cost of transgenic farm founders remains elevated. Dissemination of the traits of interest brought by a transgene cannot be as fast and simple as it is for most plants. The transgenes must therefore bring a relatively high profit to be utilizable.

Plants have little chance to transfer pathogens to humans. The same is true for transgenic plants. The case of animals is different. It cannot be excluded that transgenic animals have become more sensitive to some pathogens for unknown and unpredictable reasons. Plants may contain toxins for human. This is very unlikely with animals which are the first target of a deleterious transgene. FAO and WHO have proposed guidelines indicating the tests to be used to validate products from transgenic animals for human consumers (FAO/WHO 2003).

Plants are cultured in open fields and uncontrolled transgene dissemination may raise problems in some cases. The same is not true for most farm animals. Flying and swimming animals may colonize vast areas on earth without any possible control. This question has been studied and discussed by Muir (2004). This study reveals that fast-growing fish are more rapidly sexually mature than controls. They might thus invade biotopes like oceans. Excess of growth hormone fragilizes the animals and shortens their life. In a second step, the rapidly growing fish released in sea water might be responsible for a local extinction of the species. Although not very likely, this scenario cannot be ignored and, up to now, the regulation agencies did not accept the breeding of fast-growing fish using the current techniques. Complete isolation of fish farms or sterilization of the animals would solve the problem. Paradoxically, the biosafety agencies might authorize human consumption of fast-growing fish but not their breeding.

Animal transgenesis raises some ethical problems as embryo manipulation and transgenes themselves may reduce animal welfare (Van Reenen et al. 2001; Verhoog 2003). No simple answers can be given to these questions but the following classification may clarify the situation. Class 1 – laboratory animals: essentially used to get knowledge and not direct profit, show frequent unpredictable side-effects, used in limited number $\rightarrow$ possible tolerance towards suffering. Class 2 – animals used as sources of organs or pharmaceuticals: directly used for human health, may generate high profit, may suffer from known and reproducible deleterious side-effects, used in limited number $\rightarrow$ tolerance towards suffering on a case-by-case basis. Class 3 – farm animals: not strictly required in most cases for human survival, may generate profit, show known and reproducible deleterious side-effects, used in large number $\rightarrow$ no tolerance towards suffering.

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