Methods to Generate Transgenic Animals

Louis-Marie Houdebine

1 Introduction

Living organisms are by essence in permanent evolution. This phenomenon is relatively slow and it was probably not perceived by humans until they invented agriculture and breeding. The control of plant and animal reproduction made possible the empirical genetic selection which provided to human communities essentially all their food products, pets and ornamental plants. This led to the generation of profoundly genetically modified organisms. Carrots, tomatoes, silk worm, some dogs, etc., are unable to survive without the assistance of humans. The discovery of the Mendel laws allowed an improvement of the genetic selection. Yet, this selection remained based on spontaneous, thus random and unknown, mutations. During the first half of the last century, it appeared necessary and possible to increase the number of random mutations to enlarge the choice of genetically modified organisms corresponding to the expectations of experimenters, farmers and breeders. This was achieved by using chemical mutagens and by generating multiple intra- and interspecies hybrids. One of the most impressive examples is the creation of a new cereal, triticale, which results from an artificial crossing between wheat and rye. This new plant is currently a source of food for farm animals. All these methods are imprecise as they induce multiple unknown mutations in addition to those which are expected. Yet, these approaches were globally highly beneficial for humans. They show that the plasticity of living organisms is high and that humans have empirically learned to manipulate them successfully with limited undesirable side effects. The discovery of DNA and genes opened wide avenues for research and biotechnological applications. Indeed, the manipulation of isolated and known genes makes possible more diverse and better controlled genetic modifications.

The introduction of isolated genes into cells became a common practise in the 1970s, soon after the emergence of the genetic engineering techniques. It represented a great progress for the understanding of gene function and mechanisms of action. This technique is still widely used and it started being complemented in 1980 and 1983 by gene transfer into animals and plants respectively to generate lines of genetically modified organisms, also known as transgenic animals and plants. The first transgenic animals, mice, were obtained by microinjecting the genes into one on the nuclei

(pronuclei) of one day old embryos. This method could be extrapolated successfully to other mammals in 1985 but it soon appeared that other methods had to be used for some species. Another problem emerged rapidly. The transgenes worked and they were able to induce some phenotypic modifications in animals. The first example was the giant mice overexpressing growth hormone genes. It also appeared that the expression of the transgenes was not satisfactory and not easily controlled in all cases.

Two decades later, very significant improvements of the transgenesis methods have been obtained. Yet, the efficiency of gene transfer and the control of transgene expression remain limiting factors for the use of transgenic animals for research as well as for biotechnological applications. The generation and use of transgenic animals are not neutral as they imply the sacrifice and in some cases the suffering of animals. This paper aims at reviewing the different techniques of transgenesis and some of their possible interference with animal welfare.

2 Techniques of Gene Transfer

A transgenic organism results from an inheritable genetic modification induced by the artificial transfer of an exogenous DNA fragment. This implies that the introduced foreign gene is present in the gametes and is integrated into a chromosome to be transmitted to progeny as a host gene. To reach this goal, the foreign gene may be introduced in embryos at the first cell stage by a direct microinjection or via gametes. Alternatively, the foreign gene may be introduced in cells capable of participating to the complete development of the animal in which gametes contain the foreign gene. These different methods have been described in previous publications (Houdebine 2003, 2005) and they are summarized in figure 1.

2.1 DNA Microinjection

About 1,000 copies of the isolated foreign gene contained in 1–2 pl may be injected into one of the pronuclei of one day old mammalian embryos. This method implies a superovulation of the females followed by a mating with a male. The resulting embryos are collected the next day and microinjected with DNA. The embryos are then transferred to hormonally prepared recipient females using surgery operations. The yield of this method in mice is of 1–2 of transgenics for 100 microinjected and transferred embryos. It is lower in all the other mammalian species and very low in ruminants.

In non mammalian species, the pronuclei cannot be visualized and DNA must be injected into the cytoplasm of the one day old embryos. This relatively simple technique is efficient in most fish species but highly inefficient in chicken, in Xenopus, in some fish and in insects. For unknown reasons, the integration of the foreign DNA, thus, does occur in some species.

2.2 Use of Transposons

Transposons are short genomic DNA regions which are replicated and randomly integrated into the same genome. The number of a given transposon is thus increasing until the cell blocks this phenomenon to protect itself from a degradation of its genes. Foreign genes can be introduced into trans-

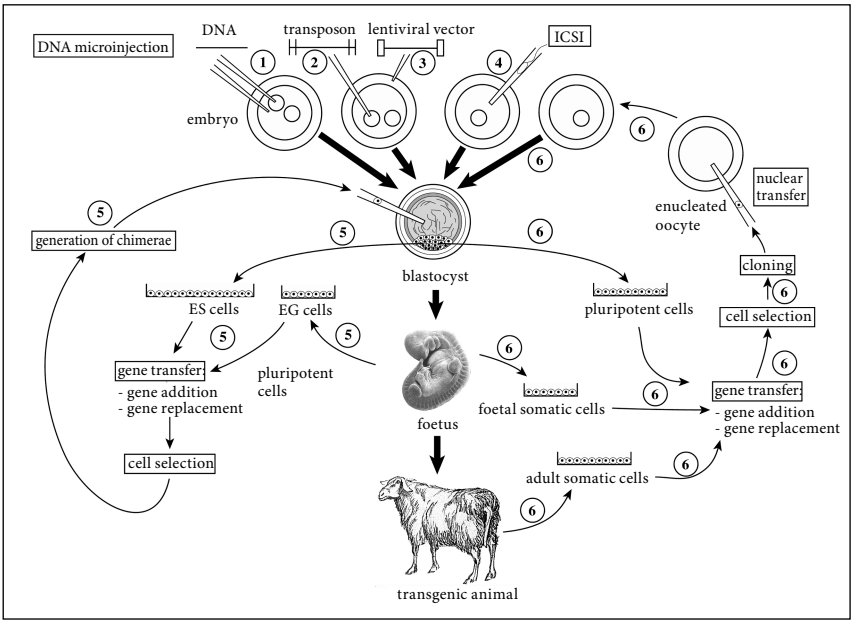

Figure 1: Different methods to generate transgenic animals: (1) DNA transfer via direct microinjection into pronucleus or cytoplasm of embryo; (2) DNA transfer via a transposon: the foreign gene is introduced in the transposon which is injected into a pronucleus; (3) DNA transfer via a lentiviral vector: the gene of interest in a lentiviral vector is injected between the zona pellucida and membrane of the oocyte or embryo; (4) DNA transfer via sperm: sperm is incubated with the foreign gene and injected into the oocyte cytoplasm for fertilization by ICSI (intracytoplasmic sperm injection); (5) DNA transfer via pluripotent cells: the foreign gene is introduced into pluripotent cell lines (ES: embryonic stem cells: lines established from early embryo, EG: embryonic gonad cells: lines established from primordial germ cells of foetal gonads); the pluripotent cells containing the foreign gene are injected into an early embryo to generate chimeric animals harbouring the foreign gene DNA; (6) DNA transfer via cloning: the foreign gene is transferred into a somatic cell, the nucleus of which is introduced into the cytoplasm of an enucleated oocyte to generate a transgenic clone. Methods 1, 2, 3 and 4 allow random gene addition whereas methods 5 and 6 allow random gene addition and targeted gene integration via homologous recombination for gene addition or gene replacement including gene knockout and knockin.

posons in vitro. The recombinant transposons may then be microinjected into one day old embryos. The foreign gene becomes integrated into the embryos with a yield of about 1%. All the transgenic insects are being generated by using transposons as vectors. Transposons also proved to be efficient to generate transgenic fish, chicken and mammals (Ding et al. 2005). Transposons are efficient tools but they can harbour no more than 2–3 kb of foreign DNA.

2.3 Use of Lentiviral Vectors

Retroviruses do not have the capacity to autoreplicate and they have to be integrated stably in the genome of the cells they infected to replicate. This explains why up to 1% of animal genomes contain degenerated retroviral genes. This property of retroviruses is being implemented to integrate foreign genes. For this purpose, the genes are removed from the genome of lentiviruses (a category of retroviruses) and replaced by the genes of interest. Viral particles are then prepared and used to transfer the foreign genes into oocytes or one-cell embryos. Safe experimental conditions have been defined to use the lentiviral vectors. This method has proved highly efficient in several species including mammals (Pfeifer 2006) and birds (Lillico et al. 2007).

2.4 Use of ICSI

More than a decade ago, it was shown that sperm, incubated in the presence of DNA before being used for fertilization, was able to transfer the foreign gene into the oocyte and generate transgenic mice. This method appeared difficult to use due to a frequent degradation of DNA (Smith and Spadafora 2005). Transgenic mice and rabbits were obtained by incubating sperm with DNA in the presence of DMSO (dimethylsuphoxide) and by using conventionnal in vitro fertilization (Shen et al. 2006). The method has been greatly improved, mainly by using ICSI (Intracytoplasmic Sperm Injection). This technique, which consists of injecting sperm into the cytoplasm of oocytes, is currently used for in vitro fertilization in humans. To transfer genes, sperms from which plasma membrane has been damaged by freezing and thawing were incubated in the presence of the gene of interest and further used for fertilization by ICSI. This method has proved efficient in mice (Moreira et al. 2007; Shinoara et al. 2007) and pigs (Yong et al. 2006). Transposon use and ICSI may be combined to increase the yield of transgenesis (Shinoara et al. 2007).

ICSI is therefore an excellent method to generate transgenic animals on condition that ICSI is possible in the considered species. One advantage of ICSI is that long fragments of DNA may be used to transfer the gene of interest. Another advantage is that foreign DNA is integrated at the first cell stage of embryos. This reduces the number of animals being mosaic for the transgene.

Another possibility consists of using sperm precursors. These cells can be collected from testes, in vitro cultured, genetically modified and reintroduced into recipient testes. This complex approach needs further studies to become utilisable.

2.5 Use of Episomal Vectors

The methods described above to transfer foreign genes rely on the integration of the DNA into the host genome. Another possibility may theoretically be to use episomal vectors capable of autoreplicating in host cells and transferring them to daughter cells. Fragments of chromosomes are being used for particular projects requiring the transfer of very long DNA fragments. These chromosomal vectors are not of an easy use and they carry a number of genes in addition of the gene of interest. These extra genes may interfere with the transgene or with the whole organism of the host.

Another possibility consists of using vectors which derive from viruses having the capacity to replicate in animal cells and to be transferred into daughter cells. Herpes viruses are naturally stably maintained as autonomous circular minichromosomes at a low copy number in animal cells. Foreign genes can be introduced into Herpes viral vectors and be maintained during cell division. This kind of vectors is generally species specific. This greatly reduces their potential use as well-known Herpes viruses are not available for all animal species.

However, episomal vectors not based on the use of viral elements are available. Such a vector proved highly efficient to transfer foreign genes into pig embryo using ICSI (Manzini et al. 2006). This vector is maintained without any selection pressure in the cells of the developing embryos but seemingly not later. This kind of vectors is therefore excellent tools to study transgene effect during early embryo development. Hence, until now, only the integration of foreign DNA into the host genome makes possible the generation of stable lines of transgenic animals.

2.6 Use of Pluripotent Cells

In some situations, the efficiency of the genetic modification is too low to be achieved by the methods described above. This is particularly the case for gene targeting (see 2.8). One possibility is to do the genetic modification in pluripotent cells further used to participate in the development of living organisms. Pluripotent cells have the capacity to participate in the development of all the organs but they cannot – such as totipotent cells are able to – each give birth to living animals. Pluripotent cells exist in the early embryos (morula and blastocysts) known as ES cells (embryonic stem cells) and in the primordial gonads known as EG cells (embryonic gonad cells). The pluripotent cells can be cultured, genetically modified, selected and transferred into morula or blastocysts. These cells participate to the development of the embryo to give birth to chimeric animals (figure 1). This

means that the organs of the animals, including sexual cells, derive from the genetically modified cells or from the recipient embryo. The offspring of these chimeric animals will harbour the genetic modification if they derive from the transplanted cells. This method is extensively used essentially in mice to inactivate (knockout) genes specifically (see 2.8).

For unknown reasons, ES cell lines have been established and used only in two mouse lines. In other lines and species, the ES loose their pluripotency and can no more give birth to chimeric animals transmitting the genetic modification to their offspring. Recent experiments have shown that the transfer of four genes into somatic cells, normally expressed in pluripotent cells, can differentiate these organ cells into pluripotent cells (Takahasha et al. 2007; Wernig et al. 2007, Nakagawa et al. 2008). These experiments open avenues for cell and gene therapy. The approach known as therapeutic cloning may no longer be necessary and pluripotent cells can potentially be obtained in different species by this method.

Recent experiments showed that the culture conditions to maintain pluripotent chicken EG cells have been found. This has greatly simplified the generation of transgenic chicken (Van de Lavoir et al. 2006; Han 2008).

2.7 Use of Cloning

The birth of Dolly the sheep demonstrated that the genome of somatic cells can be reprogrammed after being introduced into an enucleated oocyte. This generates a pseudo-embryo with a relatively low yield, capable to give birth to clones of the cell donor. This technique was initially designed to improve transgenesis efficiency in farm animals. This approach is likely to be used to accelerate genetic selection but its only real application presently is transgenesis (Robl et al. 2007). The principle of this method is described in figure 1. Genes are transferred into somatic cells which are then used to generate transgenic clones. This method has become the one most frequently used for large farm animals.

2.8 Targeted Gene Integration

All the techniques described above lead to uncontrolled but not strictly random gene integration. Foreign DNA is preferentially integrated in gene rich genome regions and its location can be precisely identified. A foreign DNA fragment can recombine very precisely with a genomic DNA region containing a similar sequence. This natural mechanism known as homologous recombination makes the precise replacement of a gene by another possible (figure 2). An active gene may thus be replaced by an inactive version leading precisely to an inactivation of the targeted gene (gene knockout). The targeted gene may also be replaced by an active gene (gene knockin). This technique therefore allows for a better controlled transgenesis reducing possible damage of the genomic DNA at the integration site and frequent side effects of the genes located in the vicinity of the transgene on the expression

of the transgene (see 3.1). Yet, this approach remains limited by the fact that the homologous recombination required for gene targeting is a rare event. The targeted integrations by homologous recombination of a foreign DNA represent 0.1–1% of the total integrations. The cells in which targeted integration has occurred must be selected and used to generate a transgenic animal. The formation of chimeric embryos using pluripotent cells (see 2.6) or the cloning technique (2.7) is required to obtain a targeted integration.

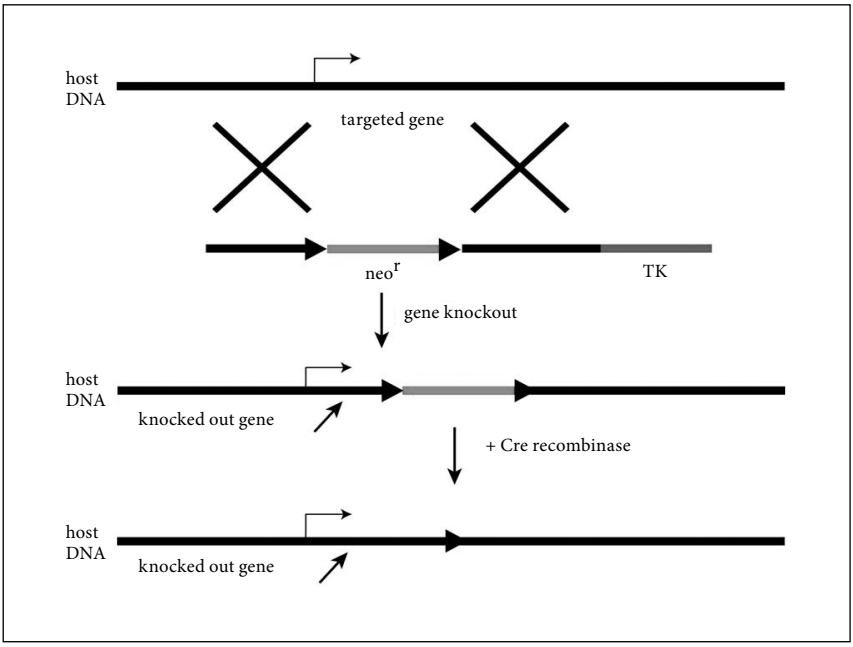

Figure 2: Targeted gene transfer via homologous recombination. A vector containing a gene for positive selection (neo R), a gene for negative selection (TK) and two sequences identical to those targeted in the genome is transferred into a cell (pluripotent or somatic cells). The homologous sequences recombine, leading to a precise replacement of the targeted genomic regions by the sequences of the vector. The neoR gene is then integrated into the targeted gene which is then inactivated (gene knockout). Cells, in which the targeted integration occurred, are selected by the addition of neomycine to the culture medium whereas the cells, in which a random integration occurred, possess the TK gene which induced a destruction of these cells in the presence of ganciclovir. The selected cells are then used to generate chimeric animals (ES or EG cells) or to generate a clone (somatic cells). If the neoR gene has been previously bordered by LoxP sequences, it may be selectively deleted by adding the Cre recombinase. The genomic region has then lost a chosen region and a LoxP sequence (about 30 bp) is the only residue of the vector. The LoxP sequence is sufficient to knock out the targeted gene.

The efficiency of homologous recombination can be markedly increased (at least 100 times) by a local break of the two DNA strands in the targeted site of integration. This can be achieved by using special restriction enzymes known as meganucleases. These enzymes, mainly found in yeast, have the capacity to cut DNA at sites which are longer than those of the classical restriction enzymes and which are usually not present in animal genomes, avoiding the degradation of DNA. The DNA sequences recognized by meganucleases must then be added to the genome of animals either at targeted sites by homologous recombination or at random sites. In the latter case, the integration sites must be validated for its capacity to allow a good gene expression before targeting the gene of interest at the meganuclease site. In practise, the recombination vector containing the gene to be transferred bordered by two DNA sequences similar to that of the genomic DNA, is introduced in the cell with the meganuclease. Engineered meganucleases capable of recognizing natural genomic DNA sequences make gene targeting at multiple sites of the genome possible (Porteus and Caroll 2005). This method, which is being developed to improve the efficiency and the precision of gene therapy, can be applied to target the integration of foreign genes into experimental animals.

In the same line, the bacterial enzyme phiC31, which is an integrase, recognizes several sites in various animal genomes and allows the efficient integration of foreign genes at the targeted sites (Rao 2008).

Several other recombination systems rely on the use of integrases such as Cre and Flp which recognize specific sites of about 30 nucleotides (LoxP and FRT respectively) which must be added to the animal genome (Baer and Bode 2001). These systems are more often used to delete a DNA region previously bordered by the LoxP or the FRT sequences (see 3.3).

3 Methods to Control Gene Expression

3.1 Use of Long Genomic DNA Fragment

The majority of DNA in somatic cells of animals is methylated on the C of CpG motifs. This results in the specific inactivation of the corresponding genes. Mammalian genomes contain about 25,000 genes and only 2,000 are active in somatic cells. About 1,000 genes, known as housekeeping genes, are active in all cell types whereas the other 1,000 genes are specifically active in given cell types to support their differentiated state. During gametogenesis and the early embryo development, DNA is heavily demethylated to blunt the gene expression programme of gonad cells from which gametes derive. After embryo implantation, DNA is progressively and selectively methylated to define which gene in the different cell types will have to be active or not in foetuses, newborns and adults. The mechanisms which control this programme of the gene expression are only partly known. It implies the contribution of multiple DNA regulatory sequences, some of them being

located far upstream or downstream of the genes. The low expression of many transgenes containing only the transcribed regions with a promoter, proximal enhancers, at least one intron and a transcription terminator, has revealed that remote regulatory regions must be involved in the control of gene expression. In a limited but significant number of cases, using long genomic DNA regions (up to 200 kb) surrounding the gene of interest greatly increases the proportion of active transgenes and also often the level of their expression (Long and Miano 2007). Interestingly and expectedly, association of classical plasmid expression vectors with long DNA fragments markedly enhances the functioning of the transgenes. This point is examplified by a milk protein gene, the WAP (whey acidic protein gene) gene. A 30 kb genomic fragment containing the pig WAP gene expressed the WAP gene in transgenic mice very poorly whereas fragments of 80 kb or 145 kb allowed a high expression of the gene, although not as a function of the integrated copy number and at various levels according to species (Saidi et al. 2007). Interestingly, coding sequences added after the WAP gene promoter in the 145 kb BAC (bacterial artificial chromosome) were expressed at a much higher level than when they were under the dependency of the only WAP gene promoter (Soler et al., unpublished data).

3.2 Specific Inhibition of Gene Expression

Transgenesis is mostly used to add foreign genetic information into an animal. Inactivating a gene in an animal is also essential, particularly for the identification of the function of the gene. Indeed, inactivating a gene may have a much stronger impact than adding a gene to an animal and thus reveal the function of the gene.

A gene is usually activated to eliminate the corresponding protein in the animal. This can be achieved by different techniques and at different levels of the protein synthesis process.

3.2.1 At the Gene Level

The data reported in section 2.8 indicate that gene knockout based on homologous recombination is a very efficient but laborious method with a major limit. The gene knockout is currently performed early in development and this event is irreversible. Experimenters may wish to prevent the expression of a gene reversibly, in a given cell type only and at chosen periods of the animal's life. Available methods make the gene knockout possible in a given cell type at a chosen moment. This technique, which has been used successfully for more than one decade, leads to an irreversible inactivation of the gene.

3.2.2 At the mRNA Level

It is possible to inhibit a mRNA specifically by adding to the cells short synthetic oligonucleotides, having a sequence complementary to that of the targeted mRNA. These oligonucleotides contain some analogues of natural

deoxyribonucleotides to enhance their stability in vivo. The binding of the oligonucleotides to the corresponding mRNA induces a degradation of the mRNA. This tool is currently used to inhibit the expression of genes in cultured cells. Oligonucleotides are also administered to patients to tentatively inhibit viral genes in order to block infections. This approach has met limited success so far.

Another possibility to inhibit a mRNA consists of introducing in a cell an anti-sense RNA generated by the transcription of the non-coding strand of the gene. The anti-sense RNA forms a double strand RNA with the mRNA which cannot be translated anymore. In practise, this method is rarely efficient, as the mRNA and the anti-sense RNA each form multiple short double strands with their own sequences. These RNA are also more or less associated with proteins. These two events prevent the easy formation of a double strand mRNA-anti-sense RNA. Hence, anti-sense RNAs meet success at most when a single region strand of the anti-sense is complementary to a single strand region of the targeted mRNA.

Ribozymes are short RNAs capable of cleaving a complementary RNA. This natural mechanism does not imply the contribution of proteins as ribozymes possess an intrinsic RNase activity. The use of ribozymes has proved disappointing in practise as, like antisense RNAs, they do not easily reach their target RNA sequence.

The discovery of interfering RNA one decade ago has profoundly improved the situation. It was unexpectedly found that a long double strand RNA can induce the degradation of a RNA having a complementary structure. Soon after, it was shown that the long double strand RNAs are randomly cut into 19–21 nucleotide fragments known as siRNA (small interfering RNA). One of the two strands of the siRNA is kept and targeted to a mRNA having a complementary sequence. This induces the degradation of the mRNA. It was also demonstrated that synthetic siRNAs transfected into cells had an RNAi effect. Soon after, the use of promoters directed by RNA polymerase III could synthesize siRNAs. In practise, a synthetic gene, containing the targeted 19–21 nucleotide sequence, followed a short random sequence and by the targeted sequence in the opposite orientation is linked to a promoter acting with RNA polymerase III (usually U6 or H1 gene promoters). The RNAs synthesized by such vectors form a 19–21 nucleotide double strand RNA separated by a loop containing the random sequence. These RNAs known as shRNAs (short hairpin RNA) are processed in cells to generate active siRNAs.

The recent discovery of the role of microRNAs has increased the possibility to use interfering RNAs. Indeed, microRNAs (miRNAs) are present in all eukaryotic cells. They are encoded by short genes expressed under the control of RNA polymerase II promoters. The primary products of these genes are 135 nucleotides RNAs which are processed and transferred to the cytoplasm where they are transformed into siRNAs. The mature miRNAs

which are fully complementary to the targeted mRNA induce a degradation of this mRNA. The miRNAs, which are only partially complementary to the targeted mRNA and which recognize a sequence located in the 3'UTR (3'untranslated region) of the mRNA, inhibit translation of this mRNA without inducing its degradation. A mammalian genome contains several hundreds of miRNA genes. Their function is essential as they control the expression of a large proportion of the genes by modulating translation of the mRNAs. On the contrary, the siRNAs described above appear more as a defence mechanism degrading exogenous double strand RNAs (e.g. some viral RNAs) as well as badly shaped cellular RNAs.

The possibility to generate transgenic animals expressing siRNAs preventing specifically the expression of a gene by degrading the corresponding mRNA or inhibiting its translation has opened avenues for the control of gene expression in vivo. This approach has met brilliant success in plants. A rapidly increasing number of transgenic plants expressing genes coding for siRNAs resistant to pathogens or having a metabolic pathway specifically blocked are available. The application of the siRNA approach is not so easy in animals for several reasons. Long double strand RNAs induce interferons and some unspecific immune reactions (Sioud 2006) and they cannot be easily used. The siRNAs are auto-amplified in plants in lower invertebrates but not in vertebrates. Moreover, the reliable expression of transgenes coding for siRNAs is more difficult in animals than in plants, as promoters directed by RNA polymerase III are generally poorly active in transgenic animals unless they are inserted into lentiviral vectors (Tiscornia et al. 2003) or associated with vectors directed by RNA polymerase II (Sawafta et al., unpublished data). It remains that the use of RNA polymerase II promoters to direct the synthesis of siRNAs offers unprecedented possibilities for experimenters and biotechnologists to modulate specific gene expression in a potent and subtle manner.

3.2.3 At the Protein Level

In most cases, the expression of a gene is achieved to suppress or inactivate the corresponding protein in cells or whole living organisms. This goal can be reached directly using different techniques. One of them consists of expressing a gene coding for an antibody specifically recognizing and inactivating the targeted protein. This kind of antibodies, known as intrabodies, must not be secreted and can even be targeted to some cell compartments to reach the intracellular proteins.

Another possibility relies on the overexpression of a gene coding for an inactive analogue of the targeted protein. The protein encoded by the gene can suppress or greatly attenuate the activity of the protein of interest by playing the role of a decoy. This was exemplified more than one decade ago. A gene coding for an inactive analogue of an insulin receptor still capable of binding the hormone allowed the generation of transgenic mice that were

no more sensitive to insulin and thus suffering from type II diabetes. This strategy is currently used in nature. This is namely the case for some hormone receptors and transcription factors which exist under different forms synthesized in the same cells in a controlled manner.

3.3. Control of Transgene Expression by Exogenous Factors

All the vectors described above and used to express transgenes contain promoters which are naturally active in the cells of the transgenic animals. This implies that the transgenes are regulated by the natural inducers of the host genes. In some cases, artificial promoters containing natural or mutated regulatory elements associated in a non natural manner are used to direct transgene expression. Transgene expressed under the direction of injected oestrogens is one example among many others. This approach is fundamentally limited. The oestrogenic inducers will stimulate or inhibit not only the transgene but a number of host genes, leading to complex and unknown side effects in the animals.

To circumvent these problems, artificial promoters, containing regulatory elements from both animal genes and bacterial genes, have been designed. The resulting promoters are active in animal cells but controlled by substances active in bacteria but not in animals. The most popular system is based on the use of the bacterial tetracycline repressor gene. In practise, the transgene becomes reversibly activated only when tetracycline is administered to the animals. A number of similar systems are available and currently used in transgenic animals with good success (Malphettes and Fussenegger 2006). These tools virtually offer the possibility to express a transgene precisely in a given cell type and at a given moment.

3.4 Deletion of Transgenes or Genomic DNA Regions

Deletion of genomic DNA regions is required in some circumstances. Conventional homologous recombination makes gene deletion, known as knockout, possible (see 2.8). Another possibility consists of using the Cre-LoxP or Flp-FRT sytems. A LoxP sequence must first be added on both ends of the fragment to delete. The presence of the Cre recombinase will then recombine the two LoxP sites leading to a deletion of the DNA fragment located between the LoxP regions. The Cre recombinase may be synthesized by the corresponding gene under the direction of a cell specific promoter. The Cre recombinase will be present and the deletion of the DNA fragment will take place only in the cells in which the promoter is active (figure 3). This process can be controlled by still more sophisticated tools. The promoter directing the Cre gene expression can be under the dependency of tetracycline. The deletion of the DNA fragment will then occur only in the chosen cell type and at the chosen moment. Another level of control can be obtained by using an engineered Cre recombinase which becomes reversibly active in the presence of an oestrogen analogue, 4-hydroxy tamoxifen.

This offers the advantage of having the active Cre recombinase for short periods of time. This prevents the non-specific action of the Cre recombinase which can recognize cryptic sites in the host genome and induce illegitimate recombinations damaging the integrity of the host DNA. The steps to use of 4-hydroxy tamoxifen-dependent Cre recombinase are depicted in figure 3.

The Cre-LoxP approach may be implemented to withdraw a given gene or a regulatory region from a genome. In the first case, the operation is known as conditional knockout. The same technique may also be used to activate a gene conditionally. In this case, a DNA sequence having an inhibitory action on transcription and bordered by two LoxP sequences may be added between the promoter of a gene and its transcription start site. The

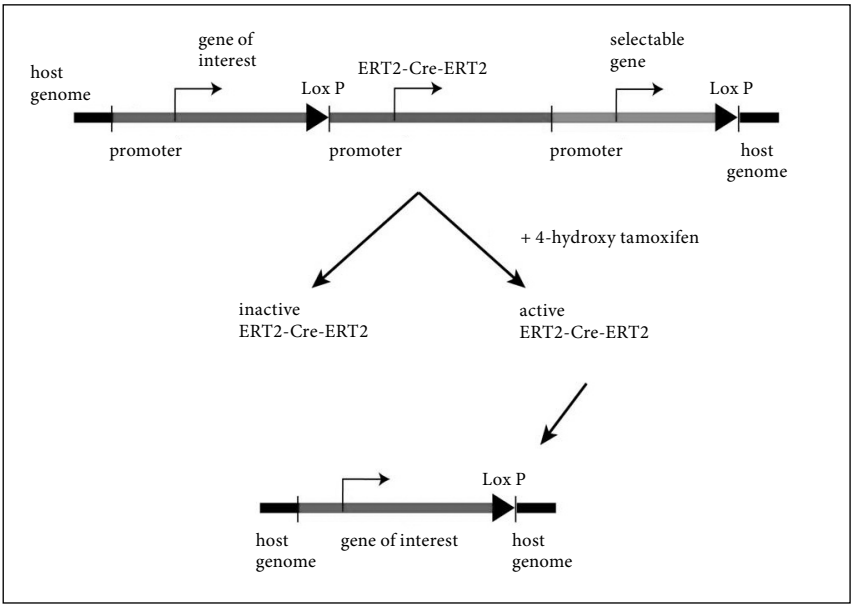

Figure 3: Elimination of the marker and selectable genes. The vector for homologous recombination (not shown here) allowed a gene knockout. The genomic targeted gene was interrupted by a DNA sequence containing a selectable gene but also the gene for a form of Cre recombinase (ERT2-Cre-ERT2 active only in the presence of 4-hydroxy tamoxifen) and two LoxP sequences. After the gene knockout, even at the next animal generation, 4-hydroxy tamoxifen may be added to embryos. This activates the Cre recombinase which recombines the two LoxP sequences leading to the elimination of the selectable gene and of the Cre recombinase gene. The remaining LoxP sequence is sufficient to knockout the targeted gene. This approach allows the elimination of the DNA sequences not necessary for the inactivation of the targeted gene and it avoids the toxic effects of overexpressed Cre recombinase.

gene will remain silent until the inhibitory sequence is deleted by the action of the Cre recombinase. Another application of this tool is the elimination of marker genes. Some of the techniques described in section 3 imply the use of marker genes or selection genes. This is the case when pluripotent cells or the cloning techniques are implemented (Houdebine 2007a). The marker genes and selection genes are not required for the action of the transgene. Their presence in the animals may have no effects, yet their elimination has been recommended by FAO/WHO and Codex Alimentarius (FAO/WHO 2007).

4 Conclusions and Perspectives

The methods to generate transgenic animals and to control transgene expression have made very significant progress during the last few years. This greatly contributes to facilitate basic research and biotechnological applications, even if the efficiency of the transgenesis techniques remains a limiting point.

The main uses of transgenic animals are presently the following ones. More than 90% of transgenic animals are used to study gene function and mechanisms of action. Many transgenic models are also generated specifically to study human diseases and to validate new medicaments (Houdebine 2007b). The possibility of grafting pig organs to humans requires transgenesis for both studying rejection mechanisms and to generate the pig donors in future (Petersen et al. 2008; Niemann, this issue). Milk from transgenic mammals and chicken egg white has started being the source of pharmaceutical proteins (Van de Lavoir et al. 2006; Houdebine 2008). A number of projects aiming at improving animal breeding are in course (Laible 2008). The most advanced project concerns salmon farming. Faster growing salmons have been obtained by overexpressing salmon growth hormone genes. This project waits for industrial development until the confinement, either physical or physiological, of these fish will become a reality (Kaputchinsky et al. 2007).

The use of transgenic animals raises specific biosafety and ethical problems. A number of research projects imply the use of dangerous animals, essentially when pathogenic organisms are being studied. These experiments are performed in appropriate confined areas and no accident due to this kind of research has been reported so far. Environmental problems are less numerous than those raised by some transgenic plants. Indeed, most farm animals are kept in confined areas and most of them have no wild partners in their neighbourhood. The consumption of products from transgenic animals is likely to become a reality in the coming years. Specific guidelines have been defined for this purpose and are ready to be adopted by Codex Alimentarius. The tests applied to transgenic plants have been

extrapolated to animals. Animals offer similar and distinct biosafety problems. Healthy animals and mainly mammals have very little chance to contain toxic substances generated by the presence of a transgene as they are themselves the first target of such substances. Compared with plants, some pathogens might proliferate more easily on some transgenic animals and be transmitted to humans.

Some problems of welfare are clearly specific of transgenic animals. Transgenesis per se may reduce animal welfare in some cases as it includes oocyte collection and embryo transfer. In some cases, the transgene can induce a specific suffering. The use of transgenesis is very diverse and the ethical problems are also diverse. It is important to note that the improvement of transgenesis techniques contributes to a diminuition of animal suffering. Indeed, an increase in transgenesis efficiency reduces the number of animals to be used for experimentation. A well controlled expression of the transgenes may also reduce their side effects. A number of projects aimed at preventing animal diseases are in course. They may contribute to the reduction of animal suffering. It may be useful to classify the different uses of transgenic animals. In a first class there are the experimental animals used for research. These animals are not very numerous in each experiment, they are not produced to make money and the effects of transgenes cannot be predicted in all cases. A second class may include animals being the source of products for the treatment of human diseases. Animals producing pharmaceutical proteins or organs for patients belong to this class. In these cases, the suffering of animals is known and a reality for a number of them. These animals may contribute to patient treatment but they may also bring substantial benefit to companies. The third class may include farm animals. Their use virtually implies a large number of animals having a known and transmittable suffering if any, with a limited impact on human survival. Tolerance towards suffering may then be the highest for the use of the first class animals, on a case by case basis for the second class animals and totally non-existent for the third class animals.

A specific problem of animal transgenesis is its possible extrapolation to humans. This extrapolation is very likely possible, especially by using gene transfer techniques such as those implementing ICSI or lentiviral vectors. It is worth noting that, as far as we know, these techniques of gene transfer have never been used in humans. Important technical problems remain to be solved to envisage transgenesis in humans. One of these problems is the control of gene integration. It must be noted that gene targeting is potentially feasible using engineered meganucleases. The major problem remains the actions of the transgenes. Many genes have complex and multiple functions in mammals. The side effects generated by a transgene cannot be fully predicted and this bottleneck may remain for a long time. Whatever happens, the major potentially acceptable application of transgenesis in humans is expected to be the protection against diseases. This implies

that the genes responsible for the diseases are known. If this is the case, it appears simpler, safer and more ethical to eliminate the embryos harbouring the faulty alleles than trying to modify them.

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