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Abstract
Gene transfer methods for generating transgenic animals follow a logical progression from gene isolation to transgene construction and gene transfer into cells. Transgenesis offers the unprecedented opportunity to generate tools to study gene function and their mechanisms of action, and has multiple biotechnological applications in the fields of medicine and food. The success of animal transgenesis was limited for many years by technical problems at key stages. One challenge is the low efficiency of foreign DNA integration, and particularly of targeted gene transfer, in some species. Another problem is the unreliability of transgene expression. Technical developments mean that these stages no longer form bottlenecks, but improvements are still needed. This review outlines the state of the art of animal transgenesis and is followed by two other reviews addressing the advances in the development of the applications, the problems of food safety, animal welfare and acceptability, including the transfer of relevant information to the public.

Riassunto
I metodi di trasferimento genico per generare animali transgenici seguono la progressione logica che va dall'isolamento del gene, alla costruzione del transgene, al trasferimento genico nelle cellule. La transgenesi offre l'opportunità senza precedenti di generare strumenti per studiare la funzione dei geni e i loro meccanismi di azione, e ha molteplici applicazioni biotecnologiche nei campi della medicina e dell'alimentazione. Il successo della transgenesi animale è stato limitato per molti anni da problemi tecnici nelle fasi chiave del processo, come, in alcune specie, la bassa efficienza di integrazione del DNA estraneo, e in particolare del trasferimento genico

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è seguito da due altri articoli che affrontano i problemi della sicurezza alimentare, benessere degli animali e l'accettabilità, ivi compreso il trasferimento delle informazioni rilevanti al pubblico.

Keywords: *transgenic animals, gene transfer, transgene expression.*

1. INTRODUCTION

Genetic selection started with the development of agriculture and plant and animal breeding, and has provided us with most of our foods, pets and ornamental plants. Conventional genetic selection is based on spontaneous mutations occurring at random sites, followed by careful observation of the resulting animals and choosing the best progenitors for further breeding. The discovery of the Mendelian laws of genetic transmission improved the efficiency of genetic selection without modifying this approach. During the last century, it became possible to increase the frequency of random mutations using chemical and physical mutagens, as well as by generating intra and interspecific hybrids. Increased variability induced by these methods benefited researchers as well as farmers and breeders. A striking example is the generation of mules by cross-breeding horses and donkeys. Still more impressive is the creation of new plant species. For example, triticale was generated through artificial cross-fertilisation of wheat and rye, and is currently being used as animal feed. The different methods of genetic selection are summarised in Figure 1.

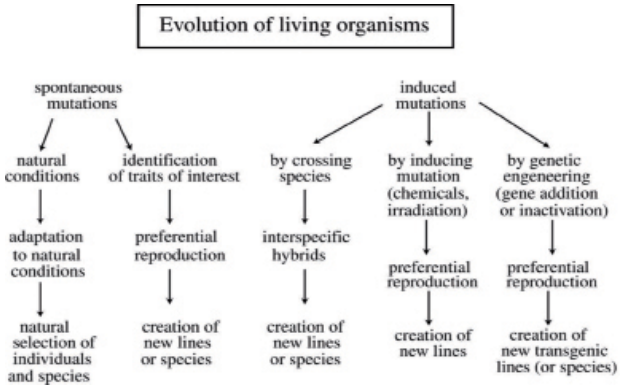

Figure 1. The different selection methods. Reproduced with permission.

However, these conventional methods are imprecise as they induce a number of random mutations in addition to those being selected. Conventional genetic selection to create models for biological or medical studies or to improve animal production relies on sexual reproduction, which involves spontaneous chromosome recombination during meiosis.

This mechanism leads to not only the selection of the undefined genes of interest responsible for favourable traits, but also to co-selection of a large number of neighbouring genes (Figure 2). Nevertheless, these different techniques, although imprecise, have been highly beneficial for human communities worldwide. They have also demonstrated the high plasticity of living organisms and, moreover, reveal that humans have empirically learned to successfully induce genetic modifications in living organisms with a limited number of undesirable side-effects. The manipulation of isolated genes allows the generation of more diverse and controlled genetic modifications (Figure 2). Transgenesis consists of artificially transferring specific genes into whole living organisms, thus transferring exogenous heritable genetic traits into living organisms.

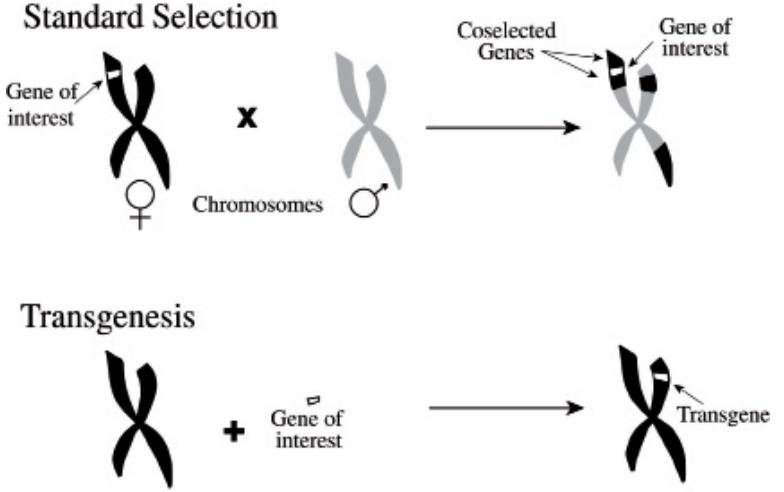

Figure 2. Impact of evolution and transgenesis on genome modification. Reproduced with permission.
The classical genetic selection relies on the recombination of homologous chromosomes during gamete formation and the random distribution of parental genes to progeny. Transgenesis provides organisms in one generation with exogenous genes having known and potentially useful properties.

This may be achieved with minimal alteration to the host genome, as judged by "-omics" studies of transgenic plants (Ricroch *et al.*, 2011). Alternatively, transgenesis can be used to improve the efficiency of a biological function. For example, the salmon *growth hormone (GH)* gene has been shown to accelerate salmon growth (Rahman *et al.*, 1998). Several copies of the *GH gene* have been transferred into animals of the same species from which the gene was originally isolated, resulting in increased growth. This process of transferring genes within a single species is known as cisgenesis and is thought to present a reduced food biosafety risk.

Data obtained in animals since 1980 and in plants since 1983 indicate that conventional selection via sexual reproduction or transgenesis both generate a similar low level of risk. The fact that the transgene originates from a foreign species does not generate a risk *per se*. Insect-resistant *Bacillus thuringiensis* (Bt) maize and salmon with accelerated growth are good examples of this. Bt toxin genes are of bacterial origin and were therefore expected to result in very little alteration of maize physiology, and this appears to be the case. In contrast, transferring extra copies of the salmon *GH* gene into other salmon to accelerate growth, an essential biological function, mimics genetic selection. Indeed, GH cisgenesis dramatically accelerates salmon growth within a single generation, whereas 20 years were required to reach the same goal using conventional genetic selection. The impact of the *GH gene* on salmon physiology is therefore much higher than the effect of the Bt toxin gene on maize physiology. Therefore, risk assessment is theoretically as necessary for salmon obtained by both conventional selection and after cisgenesis as it is for Bt maize. Yet, in practice food and animal feed obtained from all transgenic organisms are examined using sophisticated tests, whereas products obtained by conventional genetic selection are evaluated using relatively simple tests.

Other theoretical points should be taken into consideration. In general, transgenesis applications are only possible when genes with well-defined biological properties are available. Hence, biological functions that depend on multiple genes may generally be improved only via conventional genetic selection. However, it is possible that a multigenic biological function can be overstimulated by the addition of a single gene if its expression is rate-limiting. This is the case for growth, which can be greatly improved by the transfer of a single *GH gene* in species like fish, in which endogenous GH availability is limiting.

2. TECHNIQUES FOR GENERATING TRANSGENIC ANIMALS

Published techniques for generating transgenic animals (Houdebine, 2003, 2010) are summarised in Figure 3. Another recent book provides details of techniques used to generate and breed transgenic animals. Although this book focusses on mice, a number of recommendations may be extrapolated to other animal species (see Houdebine, 2011).

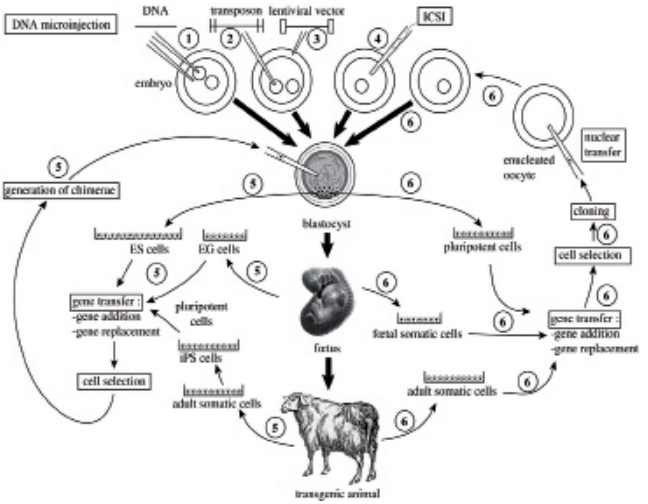

Figure 3. Different methods to generate transgenic animals.
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1) DNA transfer via direct microinjection into pronucleus or cytoplasm of embryo; (2) DNA transfer via a transposon. The foreign gene is introduced into the transposon which is then injected into a pronucleus; (3) DNA transfer via a lentiviral vector. The gene of interest introduced into a lentiviral vector is injected between the zona pellucida and membrane of the oocyte or the embryo; (4) DNA transfer via sperm. Sperm is incubated with the foreign gene and injected into the oocyte cytoplasm for fertilisation by ICSI (intracytoplasmic sperm injection); (5) DNA transfer via pluripotent or multipotent cells. The foreign gene is introduced into pluripotent cell lines (embryonic stem [ES] cells lines established from early embryo, or from cells obtained after de-differentiation of somatic cells [iPS]) or into multipotent cell lines (gonad cells [EG] 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. The multipotent EG cells containing the foreign gene are injected into embryos to generate gametes harbouring the transgene. In both cases, the transgene is transmitted to progeny; (6) DNA transfer via somatic cells nuclear transfer (SCNT). 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.

2.1. Mechanisms of foreign gene integration

Several methods are available for transferring genes into different types of cells, including embryos, stem cells, somatic cells or gametes. Foreign DNA is introduced into the cytoplasm or nucleus, including embryo pronuclei. The techniques implemented depend on the animal species and whether integration of the foreign gene is expected to be random or targeted. Foreign DNA introduced into cells often forms multimers, known as concatemers, which may lead to gene rearrangement and mutation. Several copies of the foreign gene can become organised in a head-to-tail or tandem manner when introduced into the cytoplasm. Essentially, tandem arrays are formed via homologous recombination when DNA is introduced into the nucleus. The integration of a foreign DNA fragment into the genome may occur by two different mechanisms. The most frequent process is random integration; targeted integration is much less frequent.

2.1.1. Random integration

Random integration of foreign DNA occurs through heterologous recombination triggered by the formation of imperfect hybrids between the foreign DNA and the host genomic DNA. Different lines of transgenic animals obtained by this method are therefore distinct from each other, containing different copy numbers of the foreign gene integrated at different sites in the genome. Random integration of the foreign gene may induce unpredictable local damage to the host DNA. In addition, integrated transgenes are often subject to unpredictable effects owing to local endogenous transcriptional regulatory elements. In the same way, regulatory elements within the transgenes may alter the transcription of nearby host genes. This phenomenon has not been well studied so far.

Systematic studies have indicated that transgenic mice heterozygous for transgenes rarely show abnormalities. In contrast, up to 3-10% of homozygous transgenic mice show a modified phenotype, suggesting that untargeted integration of foreign DNA may be mutagenic. It is therefore possible that transgene integration is not completely random and may preferentially occur in genes (van Reenen *et al.*, 2001; van Reenen, 2009).

2.1.2. Targeted integration

To avoid the side-effects of random integration, it is theoretically possible to target foreign DNA integration using the homologous recombination process. This universal mechanism relies on recognition between exogenous DNA and a genomic DNA sequence, which generates

molecular hybrids and leads to the precise replacement of the endogenous DNA region by the exogenous DNA fragment (Figure 4). Normally, this mechanism serves several purposes: to repair genes using the other allele as a template; to recombine homologous chromosomes during meiosis for generating gamete genomes; and to generate functional immunoglobulin genes. Homologous recombination is currently used to genetically modify bacteria and yeast. Under normal circumstances, homologous recombination is relatively rare, representing approximately 0.1-1% of the heterologous recombination events in animal cells. It cannot therefore be directly induced in early embryos using conventional techniques. Therefore, gene targeting must be done in intermediate cells, which are then used to generate transgenic animals.

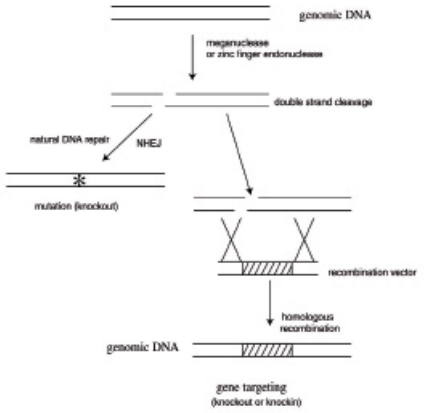

Figure 4. Gene targeting for their inactivation using random DNA repair and for gene replacement using homologous recombination.
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The introduction into a cell of a DNA fragment having part of its sequence similar to genomic DNA may replace the genomic sequence at a very low frequency. If the transferred DNA contains two sequences homologous to genomic DNA regions surrounding foreign DNA, the homologous sequences recombine (indicated by crosses) and the foreign DNA is integrated into the genome in a targeted manner. The targeted genomic gene is interrupted and thus inactivated (knock-out) by the foreign DNA. Alternatively, the foreign sequence may be a functional gene, the integration of which is precisely targeted (knock in). The homologous recombination process is enhanced up to 1000-fold when both strands of genomic DNA are locally cleaved by targeted endonucleases (meganucleases, zinc finger nucleases [ZFN] or transcription activator-like effector nucleases [TALE]). When the endonucleases are injected into the embryos without any recombinant vector, DNA break is repaired but often in a random process known as non-homologous end joining (NHEJ), generating a targeted mutation and thus a knock out. This protocol is considered as transgenesis without a transgene.

In practice, homologous recombination allows the replacement of a given genomic DNA region by an exogenous DNA fragment. Several applications of this approach are possible: (i) replacement of a functional genomic DNA sequence (generally a gene) by a non-functional DNA sequence that leads to inactivation of the target gene, in a process known as *gene knock-out* (Figure 4); and (ii) targeting a functional foreign gene into a given genomic region or the replacement of one allele by an alternative allele, in a process known as *gene knock-in*.

The frequency of homologous recombination is considerably increased when both DNA strands are cleaved at a chosen site in the genome. Classical restriction enzymes cleave genomic DNA at multiple sites, thus globally damaging the genome. To facilitate homologous recombination, nucleases should ideally recognise and cleave DNA only in the target region of the genome. This can be achieved using an endonuclease with two functional domains: one domain specifically recognises the target site in the genome and the second domain cleaves a single DNA strand in the vicinity of the binding site. Another endonuclease must be used simultaneously to cleave the second DNA strand (Figure 4). Such nucleases are known as meganucleases and are mainly found in yeast. In their native form, meganucleases cleave DNA at specific sequences that don't exist in most species. They must therefore be engineered for specific DNA cleavage at alternatives sites to induce homologous recombination. Alternatively, it is possible to generate fully engineered fusion nucleases, known as zinc finger nucleases (ZFN). These enzymes contain a zinc finger region, which specifically recognises the chosen genomic site, and a common non-specific endonuclease, Fok1 (Figure 4). A third particularly attractive possibility is to generate fusion enzymes in which the Fok1 domain is associated with specific DNA-recognition domains derived from the transcription activator-like effector (TALE) family of plant effectors. The resulting nucleases, known as TALE nucleases, can be engineered to target virtually any genomic site (Boch, 2011).

The introduction of recombination vectors into cells along with these nucleases allows targeted gene replacement (Figure 4). Recent publications have shown that both targeted knockout (Rémy *et al.*, 2010) and targeted gene integration (Meyer *et al.*, 2010) can be directly achieved in embryos.

Spontaneous targeted gene knockout has also been shown to occur following nuclease microinjection in the absence of foreign DNA. In this case, cleavage of both DNA strands induces the DNA repair mechanism

known as non-homologous end joining (NHEJ). DNA repair by this process is often imperfect and results in mutations that are similar to gene knockout. This process, known as transgenesis without a transgene, can be efficiently conducted in embryos (Rémy *et al.*, 2010). Moreover, ZFN can be used to efficiently integrate foreign genes bordered by ZFN cleavage sites into genomic sites that are also specifically cleaved by the ZFN (Orlando *et al.*, 2010). These new tools are expected to have a strong impact on transgenesis applications.

2.2. Techniques for DNA transfer

To generate transgenic animals, foreign DNA must be present in one-cell embryos. This can be done by introducing DNA into the embryo or into other cells involved in the reproduction cycle.

2.2.1. DNA microinjection

Gene constructs (1000-5000 copies) in 1-2 picolitres can be injected into a pronucleus of one-cell mammalian embryos. This technique requires a large number of embryos obtained by superovulation of females followed by either mating with a male or by artificial insemination. Microinjected one-cell embryos must be transferred to hormonally-prepared recipient females. In mice, this technique yields 1-3 transgenic animals from 100 transferred embryos. Despite being laborious, this technique remains the most commonly used in mice and rabbits. However, the rate of DNA integration after microinjection into pronuclei is lower in all the other mammalian species and it is particularly low in ruminants.

When DNA is microinjected into pronuclei, approximately 30% of the transgenic founders transmit their transgene at a lower than expected rate. This lack of conformation to the laws of Mendelian inheritance means that at least 30% of the founders are mosaic for the transgene. In these mice, integration of the foreign gene occurs not in the first cell, but later at the two- or four-cell stage of embryo development. Therefore, the transgene is not present in all gametes of these founders. As expected, the proportion of transgenic animals in the next generation is as predicted by Mendelian inheritance (Echelard, 1997). For the same reason, approximately 1% of transgenic founders do not transmit the transgene, indicating that they are so highly mosaic that the transgene is rarely present in their gametes.

In non-mammalian species, pronuclei cannot be visualised, as the embryonic cell is embedded in the opaque vitellus. In these species, large amounts of DNA (several millions of copies in a few nanolitres) have therefore to

be microinjected into the cytoplasm of one-cell embryos. This relatively simple technique has proved efficient in several fish species (Maclean, 2003) but, for unknown reasons, the rate of integration is extremely low in various species such as chicken, *Xenopus*, some fish species and insects.

In lower vertebrates and invertebrates, independent integration events often take place within the same embryo during the first days of development. Several reproduction cycles are then needed to segregate the different copies of integrated DNA until lines can be obtained that contain transgene integration at a single site.

Direct DNA microinjection into embryos is therefore a useful technique, but only for some species. Other methods of gene transfer are still under development.

2.2.2. Transposons

Transposons are short genomic DNA regions (about 1-3 kb) that autoreplicate and are randomly integrated as multiple copies within the same genome. Integration is achieved by the integrase enzyme encoded by the transposon itself. Foreign DNA can be introduced into the transposon to replace the *integrase* gene, and the recombinant transposon can be microinjected into one-cell embryos along with the transposon integrase enzyme. Using this method, the foreign gene can be integrated into the embryo genome with an efficiency of 1% or more. All transgenic insects are now generated using specific transposon vectors. Some transposon vectors have also proven capable of generating transgenic chicken, fish and mammals (Dupuy *et al.*, 2002; Ding *et al.*, 2005). Some engineered transposons can transfer foreign genes efficiently in multiple species. The efficiency of transposon-mediated gene transfer has recently been greatly enhanced, and the transfer of DNA fragments as long as 120 kb is now possible with a high efficiency (Moisyadi *et al.*, 2009; Suster *et al.*, 2009; Sumiyama *et al.*, 2010).

2.2.3. Lentiviral vectors

Retroviruses do not have the capacity to autoreplicate: they must be stably integrated in the genome of host cells to replicate. This property has been exploited to integrate foreign genes into various cell types, including embryos. For this, viral genes are deleted and replaced by the gene of interest. Viral particles are then prepared in cultured cells and introduced into oocytes or one-cell embryos (Ritchie *et al.*, 2009). Lentiviruses (a category of retroviruses) are used preferentially as they can efficiently

integrate genes into non-replicating cells. In lentiviral vectors, the viral envelope is replaced by a vesicular stomatitis virus protein that recognises the plasma membrane of most cell types. Currently, lentiviral vectors are being used successfully and safely in a variety of species.

2.2.4. Intra-cytoplasmic sperm injection (ICSI)

Intra-cytoplasmic sperm injection (ICSI) is an efficient *in vitro* fertilisation technique currently used in humans that relies on the injection of sperm into the cytoplasm of oocytes. To transfer foreign genes using ICSI, the sperm plasma membrane is first damaged by freezing/thawing and inactivated sperm are then incubated with DNA and used for fertilisation. This method is efficient in mice (Moreira *et al.*, 2007; Shinohara *et al.*, 2007) and pigs (Yong *et al.*, 2006); however, its use in other mammalian species is limited by the relative inefficiency of the ICSI technique in these species.

2.3. Use of intermediate cells

The gene transfer techniques described above are often too inefficient (e.g. gene targeting) to be used directly in embryos. Instead, gene modifications can be carried out in intermediate cells that can then participate in the development of an embryo and transmit the genetic trait to progeny. Several cell types are currently used for this purpose (see Figure 3).

2.3.1. Pluripotent cells

One possibility is that genetic modifications can be made to pluripotent cells, which then contribute to the development of chimeric embryos. By definition, pluripotent cells have the capacity to participate in the development of all organs, including gametes.

Pluripotent cells exist in early embryos (morula and blastocysts), where they are known as embryonic stem (ES) cells. ES cells can be maintained in culture, genetically modified, selected and injected into recipient embryos at the morula or blastocyst stage, as well as in one-cell embryos, and then participate in embryonic development, leading to the production of chimeric transgenic animals (Figure 3). All cells of the chimeric animals, including the gametes, derive from either the pluripotent genetically modified cells or the recipient embryo. Therefore, a proportion of the gametes harbour the genetic modification, which can therefore be transmitted to progeny.

At the end of the 1980s, ES cells were used for the first time to create genetically modified animals (mice) (Capecchi, 1989; Bronson & Smithies,

1994). For the next two decades, ES cells from two mouse lines were the only cells that could be used for this purpose. It was discovered that ES cells have a strong propensity to differentiate *in vitro* as well as *in vivo*, and the resulting genetically modified ES cells can lose their pluripotency and become unable to contribute to the formation of chimeric animals capable of transmitting their genetic modification to progeny.

An improved understanding of the pluripotent state showed that a few genes that are always expressed in ES cells are required to maintain pluripotency. Additional studies led to the identification of chemical compounds that can control the expression of these pluripotency genes. ES cells can now be obtained by adding these compounds to the culture medium of rat embryonic cells, thus enabling the development of rat *gene knock-out* and *knock-in* procedures (Hamra, 2010). However, so far this approach has proven to be inefficient in other species and chemical compounds that can stimulate regeneration from ES cells in other species remain to be found.

Interestingly, the transfer of three or four genes normally expressed in pluripotent cells into somatic cells can induce their dedifferentiation into pluripotent cells. These *induced pluripotent cells* (iPS) have similar properties to ES cells (Takahashi *et al.*, 2007; Wernig *et al.*, 2007; Nakagawa *et al.*, 2008; Pera & Hasegawa, 2008). In addition, it was recently shown that the transfer of two microRNAs into somatic cells can induce their transformation into iPS cells (Anokye-Danso *et al.*, 2011). These experiments open important new avenues for cell and gene therapy. iPS cells have been obtained in different species, and are thus expected to be a new tool for generating transgenic animals in species for which ES cells are not available (Figure 3) (Yiu-Fai Lee & Kent Lloyd, 2011).

2.3.2. Primordial germ cells (PGC) and testis stem cells

Primordial germ cells (PGC) are embryonic multipotent cells involved in the development of gonads and the formation of gametes. PGC have been obtained from different species but cannot so far be used to generate healthy offspring. Recent experiments have demonstrated that chicken PGC can be cultured under experimental conditions that maintain their multipotency, thus establishing stable embryonic gonad (EG) cell lines. To optimise the chance of EG cells colonising an embryo, EG cells containing a gene of interest were injected into an early embryo in which the majority of the cells had been destroyed by irradiation. This approach proved very successful and has greatly simplified the generation of transgenic chickens

(Van de Lavoir *et al.*, 2006a, b; Han, 2009).

In mice and a few other species, testicular sperm precursors can be isolated, cultured and genetically modified. They can also be induced to partially differentiate *in vitro* and then transplanted into recipient testis to give functional sperm capable of generating transgenic animals by fertilisation. Sperm cell precursors can also be genetically modified *in situ* using either viral vectors or transposons (Takahashi *et al.*, 2007; Han, 2009; Carlson *et al.*, 2011). These promising methods are still at the developmental stage and they are not currently used to generate transgenic animals.

2.3.3. Somatic cells and somatic cell nuclear transfer (SCNT)

The birth of Dolly the sheep demonstrated that somatic cells can be dedifferentiated following their introduction into enucleated oocytes, thus generating pseudo-embryos capable of developing into clones of the cell donor. This technique was developed to improve transgenesis efficiency in farm animals (Schnieke *et al.*, 1997; Robl *et al.*, 2007). Genes can be transferred into cultured somatic cells and used to generate transgenic clones (Figure 3). This simple and efficient method of transgenesis is now frequently used in farm animals.

Interestingly, it has been shown that somatic cell nuclear transfer (SCNT) does not induce mutations in the genome of the clone (Murphey *et al.*, 2009). However, in cows and sheep, SCNT gives rise to a number of abnormal foetuses; this problem is less frequent in pigs and goats (EFSA, 2008; Houdebine *et al.*, 2008). The abnormal foetus development results from an incomplete programming of the somatic cell genome. This leads to abnormal epigenetic modifications and to erroneous gene expression.

SCNT is currently the most frequently used technique to generate transgenic ruminants and pigs. The emerging gene transfer techniques depicted in this chapter suggest that the use of SCNT to generate transgenic farm animals may become less necessary in the coming decades. The deleterious side effects associated with the use of SCNT in animals may thus be reduced accordingly (Houdebine, 2010).

3. VECTORS FOR TRANSGENE EXPRESSION

In the early 1980s, the very first transgenes were expressed and in some cases were able to induce phenotypic modifications in animals, for example giant mice were obtained in 1982 (Palmiter *et al.*, 1982) by overexpressing foreign *GH gene*s. It soon appeared that transgene expression was often far from satisfactory. Thus, for a number of years, gene constructs were obtained and used empirically, sometimes with limited efficiency. The strategy of researchers was, and often still is, to generate a relatively large number of transgenic lines (in mice and other species) and to keep only those in which the transgene is transmitted at a sufficiently high rate and expressed as expected. This strategy is not optimal for transgenesis in large animals or when finely tuned transgene expression is needed.

3.1. Basic vectors

In animals, gene expression is under the control of multiple regulatory sequences located proximal to the transcriptional start site, known as the promoter, and also by remote enhancers and insulators (Figure 5). Insulators are not clearly defined regulatory elements. One insulator role is to maintain chromatin in an open configuration in regions of specific genes expression. Insulators can also prevent the interaction of specific gene regulatory elements with neighbouring genes. Through the formation of loops, regulatory elements are concentrated at a site known as a hub, located in close proximity to the promoter, thus forming a transcriptional complex that modulates gene expression. Generally, only the proximal regulatory elements are present in conventional vectors.

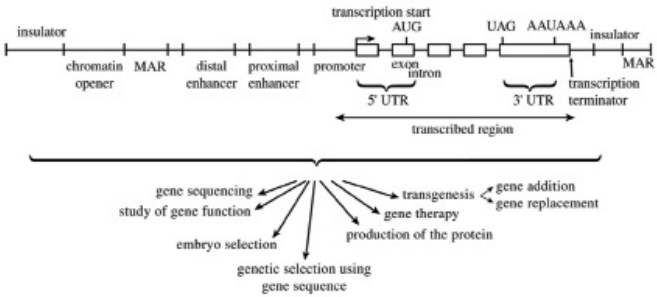

Figure 5. Organisation of animal genes including their regulators and the different applications of identified genes, including transgenesis.
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Empirical strategies for gene construction have made transgene expression more predictable, reliable and efficient (Houdebine, 2009a). The following recommendations have been proposed for optimising conventional transgene expression:

a) Evaluate the efficiency of the construct by transfecting it into cultured cells in which the promoter of the construct is active

b) Check that the sequence of the construct is correct;

c) Make sure that no part of the coding sequence of the construct is deleted by cryptic splicing. This can be checked by northern blotting or reverse transcriptase PCR. If so, remove (delete or mutate) the cryptic splicing (donor and acceptor) site(s) from the construct;

d) Add at least one intron, preferably upstream of the cDNA, to prevent nonsense-mediated decay (NMD; see below) and choose introns with good splice site consensus sequences and splicing enhancers (Mersch *et al.*, 2008). The second intron of the rabbit β-globin is recognised as being suitable for transgene expression;

e) Make sure that mRNA encoded by the transgene is not degraded by a NMD mechanism. This phenomenon occurs when the donor splice site of an intron downstream of the translated region is farther than 50 nucleotides from the stop codon (Chang *et al.*, 2007);

f) Make sure that the 3'UTR does not contain an AU-rich region containing an AUUUA motif, which induces mRNA degradation in quiescent cells (Beelman & Parker,1995);

g) Use a short AU-rich 5'UTR that is not less than 80 nucleotides to avoid formation of a stable GC-rich secondary structure. The 5'UTR must not contain initiation codons within the consensus Kozak sequence;

h) Make sure that the initiation codon is within the Kozak consensus sequence GCCA/GCCAUGG (Kozak, 1987);

i) Reduce the overall GC content of the construct and particularly CpG motifs in the region preceding and following the transcription start point (Gaszner & Felsenfeld, 2006);

j) Add one, or preferably two copies in tandem, of the 5'HS4 insulator from chicken β-globin locus upstream of the promoter-enhancer region and optionally also after the transcription terminator (Gaszner & Felsenfeld, 2006; Giraldo *et al.*, 2003);

k) Use a strong transcription terminator, e.g. from rabbit or human β-globins or from human or bovine *GH gene*s;

l) Add an mRNA stabiliser, such as the one present in the 3'UTR of β-globin (Chkheidze *et al.*, 1999);

m) Remove sequences of the transcribed region of the construct (mainly in the 3'UTR) that may be recognised by natural miRNAs of the transgenic host;

n) Use as vectors long genomic DNA fragments cloned in a bacterial artificial chromosome (BAC) containing the promoter chosen to express the transgene, and introduce the construct (without a promoter) or cDNA into the BAC downstream of the promoter, for example after the first intron (Long & Miano, 2007);

o) In bicistronic mRNA, preferably put the internal ribosome entry site (IRES) 80 nucleotides downstream of the termination codon of the first cistron to favour the expression of the second cistron (Attal *et al.*, 1996). Alternatively, and preferably, use the 2A peptide-based system (Carey *et al.*, 2010); and

p) Optimise codon usage, especially if the cDNA is not mammalian. This modification and others within the construct may require complete chemical synthesis of the cDNA or of the entire construct.

3.2. Vectors for finely tuned transgene expression

Different methods, described elsewhere in detail (Houdebine, 2003, 2009b), have been established to express transgenes in a controlled manner.

3.2.1. Gene targeting by homologous recombination

Gene targeting was originally performed using spontaneous homologous recombination (Capecchi, 1989). This method was greatly improved by generating local cleavage of both strands of the genomic DNA using engineered meganucleases, ZFN or TALE nucleases. These tools considerably facilitated gene knock-in or knockout by gene replacement, as well as by NHEJ (see Section 2.1.2).

3.2.2. Conditional knockout

Gene knock-out is often carried out in embryos or ES cells, but may generate side-effects such as early death of the embryos. To obtain a better understanding of the role of a gene in a given cell type, it may be preferable to knock it out only in the cell type of interest and at the chosen stage of the animal's life. To reach this goal, a conditional knockout may be used. This technique relies on the use of a specific recombinase, such Cre and Flp, that recombines specific short DNA sequences, such as LoxP and FRT, respectively. One of these short DNA sequences may be added either side of the genomic DNA region to be deleted. Addition of the relevant recombinase then induces recombination and elimination of the internal DNA sequence. Recombinase expression may be controlled by tissue-specific promoters, but also by exogenous inducers such as doxycycline (see below). Moreover, the Cre recombinase has been engineered to be specifically activated by 4-hydroxytamoxifen. Using this system, gene knockout is induced by administering 4-hydroxytamoxifen to the animals (Figure 6). These combined techniques offer a double-lock mechanism to control gene excision and thus improve the conditional knockout system.

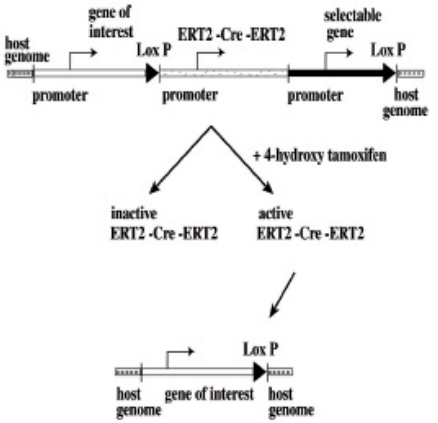

Figure 6. Tools for the conditional gene knock out.
Reproduced with permission.
The Cre recombinase can delete the region bordered by two LoxP sequences. The control of Cre gene expression and the activation of the engineered Cre recombinase by 4-hydroxy tamoxifen allow the specific induction of gene *knock-out* in chosen tissue and at a chosen stage of animal life.ge of animal life.

3.2.3. Transgene induction by exogenous factors

The use of endogenous molecules such as hormones to control transgene expression may induce a number of side-effects because many host genes are sensitive to the hormones used. To overcome this problem, artificial promoters have been constructed that rely on the use of control elements from unrelated species such as bacteria. One such system uses the bacterial tetracycline repressor fused to a eukaryotic transcription stimulator, such as tetracycline in the tet-on system, and transcription inhibitors such as KRAB. In the example shown in Figure 7, the inducer given to the transgenic animal (doxycycline, a tetracycline analogue) binds the transcription stimulator tet-on sequence, leading to gene expression. Addition of the KRAB inhibitor in the absence of doxycycline prevents gene expression.

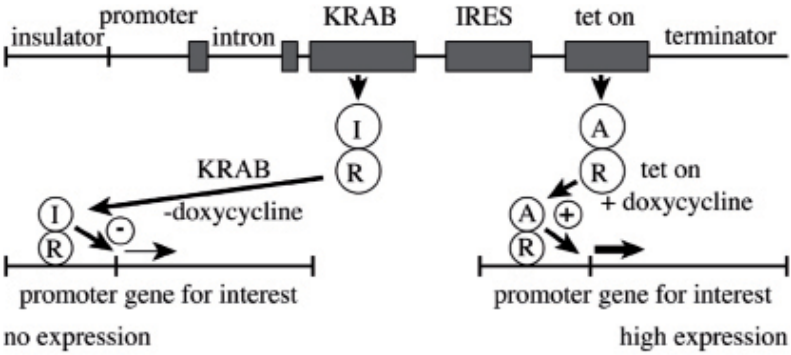

Figure 7. Controlled transgene expression by an exogenous inducer, doxycyclin. Reproduced with permission.
In the absence of doxycycline, the gene is not induced and repressed by KRAB. In the presence of doxycycline, the gene is derepressed and activated by tet-on.

3.2.4. Post-transcriptional gene inhibition

Inactivation of mRNA is currently achieved by siRNA-mediated knock-down using transgenes encoding interfering RNA, shRNA or microRNA. In this system, gene knockdown results from two independent mechanisms, both of which depend on recognition of the mRNA sequence by the interfering RNA: one mechanism leads to mRNA degradation and the other induces reversible inhibition of mRNA translation (Moazed, 2009).

4. CONCLUSION

Despite significant improvements in several transgenesis methods, the inefficiency of gene transfer and the lack of control of transgene expression remain limiting factors for the optimal use of transgenic animals in research as well as in biotechnological applications. Important improvements to these methods are being developed (Houdebine, 2010).

The use of genetically modified (GM) animals for food production remains limited compared to the widespread use of GM plants, and will probably remain so for years. Genetic modifications to farm animals are slower, more costly and more laborious than those in plants. Moreover, dissemination of transgenes throughout populations (i.e. herds or fields) is much slower and more costly in farm animals than in plants. Furthermore, possible unintended effects concerning animal health and welfare must be investigated and may trigger public debate, as is the case for GM plants; a prospect that may deter potential producers. Most domestic animals are bred in confined areas and the development of transgenic versions would raise no particular environmental problems. In contrast, the uncontrolled dissemination of transgenes is possible in flying and swimming animals. However this does not mean that the spread of transgenes implies risks *per se*. As with plants, each case should be assessed individually.

The acceptability of transgenic animals as a food source increasingly appears to be a major challenge in the EU, but also in the USA and elsewhere. USA communities involved in the biotechnological applications of transgenic animals have produced a document summarising the engagement of the different actors in this theatre (BIO, 2009). The EU supports the PEGASUS project, which aims to provide European consumers with multidisciplinary information on projects using transgenic animals (www.projectpegasus.eu).

The use of transgenic animals and their acceptability by consumers are discussed in the following two reviews.

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