2 The technology of pharming

2.1 Recombinant pharmaceutical proteins – the advent of biotechnology

In 1977 scientists succeeded in introducing the first human gene into a microorganism in order to produce a genetically engineered human protein. This "advent of biotechnology" took place only one decade after the discovery of the genetic code, which describes the connection between genes and the formation of proteins. The production of proteins by genetic engineering involves the incorporation of a foreign (often human) gene into an organism's or a cell's own genome. The genetically modified living system can then express so-called 'recombinant' proteins – it becomes a living "protein expression system". Well-established expression systems for pharmaceutical proteins include fermenter-grown genetically modified *Escherichia coli*, baker's yeasts *(Saccharomyces cerevisiae)* or Chinese hamster ovary (CHO) cell cultures. Proteins are linear chains of amino acids. Information about the sequence of each protein is encoded by the DNA of a gene. Expression of a protein commences when a gene is copied (transcribed) from a point termed the promoter, producing a messenger molecule or RNA. RNA is then processed to remove the non-coding regions or introns, and transported to the protein synthesis machinery. Here it is read (translated) and determines the amino acids added to a new protein molecule.

Different cells require different proteins and therefore express different genes. The expression of a particular gene is controlled by the interaction of factors within the cell and DNA sequences associated with the gene. These regulatory elements include the promoter and others termed enhancers (see figure 2.1).

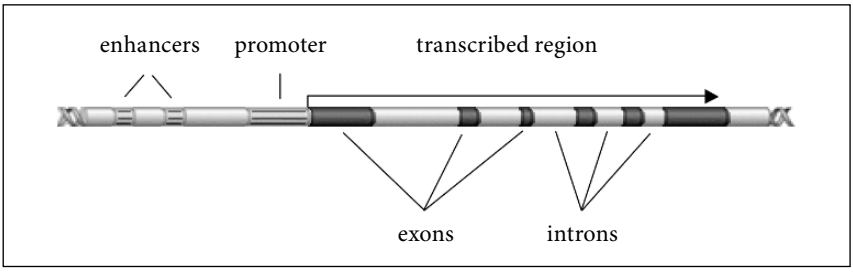


Figure 2.1: The basic structure of a eukaryotic gene

The basics of protein synthesis are highly conserved between living organisms, and this has allowed the successful transfer and expression of genes between widely different species. However many proteins require additional steps, termed post-translational modification, before they are fully functional. The types of post translational modification vary considerably between species and between cell types. The addition of sugar side chains to amino acids, called glycosylation, provides an example. The addition of complex chains of sugar molecules linked to asparagine residues in the protein chain (N-linked glycosylation) is important for the correct folding and stability of many mammalian proteins. Most bacteria are unable to glycosylate asparagine and this is a primary reason for choosing eukaryotic expression systems, including transgenic animals and mammalian cell cultures. However, the range of possible post-translational protein modifications required for protein function is very large and includes: propeptide cleavage, multichain assembly, disulphide bonding, phosphorylation, hydroxylation, amidation, methylation, hydroxylation, γ-carboxylation, acylation and lipid attachment. The repertoire of modification enzymes varies considerably between mammalian and plant tissue types. Ideally, the processing capability of the producing cells should match the requirements of the desired protein, or be readily modifiable to carry out the appropriate processing.

Humulin®, the first recombinant protein for pharmaceutical use, received marketing authorization in the USA in 1982[1]. Humulin® is recombinant human insulin produced by the bacterium *Escherichia coli*. Because of the complexity of the structure of insulin, it is not possible to synthesize it chemically. Therefore, before the development of Humulin® diabetes mellitus patients were treated with bovine or porcine insulin that was extracted from the pancreatic tissue of slaughtered cattle and pigs.

Pharmaceuticals that cannot be synthesized chemically, but rather have to be produced by transgenic living cells or isolated from biological material (for example blood donations, animal tissues) are called biopharmaceuticals. They are therapeutic proteins, with hormones and monoclonal antibodies as the most important examples, or nucleic acid-based drugs. Most biopharmaceuticals today are modern biotechnological medicines, many of which are based on proteins produced by genetic engineering[2].

---

1 FDA 1982.
2 Walsh 2003; Walsh 2006.

2.2 Plants as a production platform for recombinant biopharmaceuticals

In the past decade, plant-based expression systems have emerged as a possible alternative for the large-scale production of recombinant proteins. The major reason for the development of transgenic plants for the production of biopharmaceuticals was the expectation that costs of large-scale production would be comparatively low. This has turned out to be true for proteins that can be produced at high yields. For example, recombinant avidin was produced in maize at 20 % of total soluble seed protein[3]. Then the yield of one bushel of maize was equivalent to the total yield from one tonne of chicken eggs – the natural source of avidin. This case demonstrates the potential for cost reduction. Another important reason for the development of alternative platforms for the production of biopharmaceuticals was the hope of producing biopharmaceuticals that, so far, had to be isolated from biological material since they were too complex to be produced by recombinant microorganisms or cell culture. In addition, the risk of transmission of human pathogens via the product is minimized since plants are, in contrast to for example donated blood, not a source of human pathogens.

The first pharmaceutically relevant protein made in plants was human growth hormone, expressed in transgenic tobacco in 1986[4]. In this study the hormone was expressed as a fusion with the *Agrobacterium* nopaline synthase enzyme. Since then, many other human proteins have been produced in an increasingly diverse range of crops. In 2006 Dow AgroSciences received the world's first regulatory approval for a plant-made vaccine for animals by the United States Department of Agriculture (USDA)[5]. It is a vaccine against Newcastle disease, which infects poultry. The vaccine is produced in genetically engineered cells from non-nicotine-producing tobacco plants and has to be administered by injection. Currently the commercialization of the chicken vaccine is not planned, since the market is already crowded. Instead, the company sought USDA approval to prime the regulatory process for other animal drugs produced in the same way[6]. Table 2.1 provides examples of biopharmaceuticals produced in transgenic plants.

In broad terms the development and production of biopharmaceuticals in transgenic plants comprises the following steps: genetic engineering of the gene construct, transformation of the host plant (section 2.2.1), cultivation (section 2.2.4) and purification of plant-derived recombinant biopharmaceuticals (section 2.2.5). For the overall process see figure 2.2.

---

3 Hoot *et al.* 1997; Masarik *et al.* 2003.
4 Barta *et al.* 1986.
5 Katsnelson *et al.* 2006.
6 www.dow.com (October 2006).

Table 2.1: Some examples of biopharmaceuticals produced in transgenic plants[7]

Protein	Host plant	Company/ Organization	Indication/ application	Development stage
Animal vaccine	tobacco cells	USA, Dow AgroSciences	Newcastle disease in chicken	approved by USDA 2/2006
Enzyme, Glucocerebrosidase	carrot cells	Israel, Protalix Biotherapeutics	Gaucher disease	phase 3
Monoclonal antibody	tobacco	USA, Planet Biotechnology,	prophylaxis of caries	phase 2
Enzyme, gastric lipase	maize	France, Meristem Therapeutics	Cystic Fibrosis	phase 2
Antibody, cancer vaccine	tobacco	USA, Large Scale Biology	non-Hodgkin Lymphoma	phase 2
Alpha-Interferon	duckweed	USA, Biolex	hepatitis C	phase 2
Antigene	potato	USA, Arizona State University	hepatitis B	phase 2
Human intrinsic factor	*Arabidopsis*	Denmark, Cobento Biotech	vitamin B12 deficiency	phase 2
Antibody	tobacco	USA, Planet Biotechnology	cold caused by Rhinoviruses	phase 2
Insuline	safflower	Canada, SemBioSys Genetics Inc.	diabetes	phase1
Vaccine	lettuce	Poland, Polish academy of science	hepatitis B	phase 1
Vaccine	potato	USA, Arizona State University	Norwalk virus	phase 1
Vaccine	spinach	USA, Thomas Jefferson University, Philadelphia	rabies	phase 1
Lactoferrin	maize	France, Meristem Therapeutics	dry eye syndrome, gastro-intestinal infection	phase 1
Vaccine	potato	USA, Arizona State University	diarrhoea	phase 1
Vaccine	maize	USA, Arizona State University	diarrhoea	phase 1
Vaccine	maize	USA, ProdiGene	diarrhoea	phase 1
Alpha-Interferon	duckweed	USA, Biolex	hepatitis B	phase 1
Monoclonal antibody	not announced	USA, Planet Biotechnology	reducing adverse effects of chemotherapy	phase 1

[7] Data based on Marschall 2007, Fox 2006 and Sauter 2005.

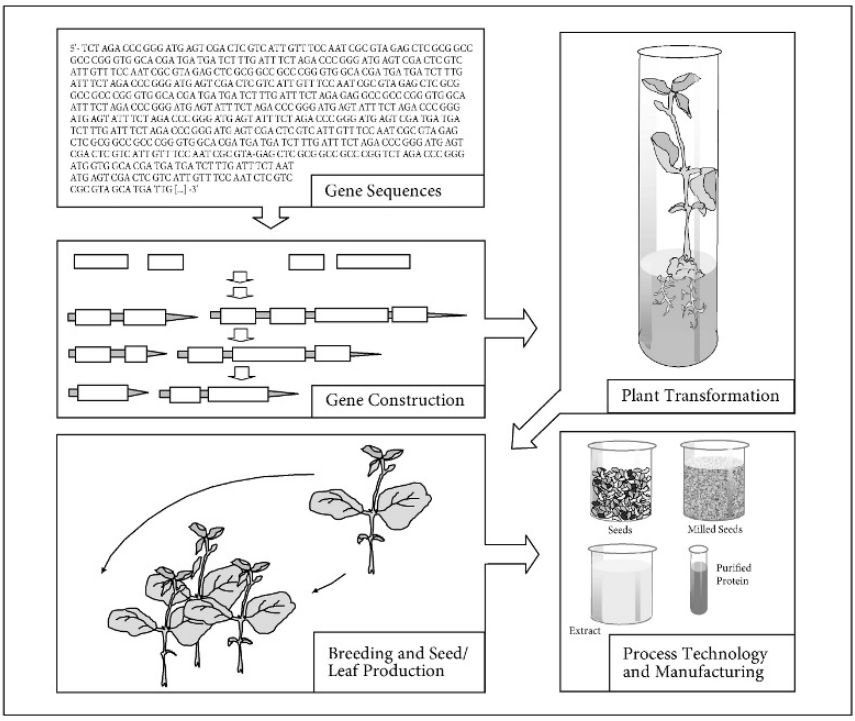


Figure 2.2: An outline of the overall process of plant pharming

2.2.1 Genetic engineering of the host plant

2.2.1.1 Gene constructs

The first step in the construction of a transgene plant for the production of biopharmaceuticals is the identification of the gene that codes for the desired protein, and subsequently the sequencing and isolation of that gene. The advances of genomics (the study of an organism's entire genome) and proteomics (the large-scale study of proteins, their structures and functions) have accelerated these steps greatly and in addition have lead to the development of new pharmaceutical applications. Once the sequence of the gene (or genes) coding for the protein is at hand an appropriate expression construct has to be developed. The expression construct needs to serve different tasks.

One main aim of plant pharming is the production of recombinant proteins at high yields. To achieve this, expression construct design seeks to optimize all stages of gene expression, from transcription to protein stability. Expression constructs are chimeric structures, in which the transgene is flanked by various regulatory elements known to be active in plants. Only by the addition of these regulatory elements is the recombinant gene recognized by the molecular machinery of the host plant and subsequently synthesized. For high expression levels, the two most important elements are

the promoter (a sequence needed to "switch" the expression of a gene on) and the polyadenylation sites which are often derived from the 19S and 35S transcripts of the cauliflower mosaic virus (CaMV)[8]. The CaMV 35S promoter is now the most popular choice in dicotyledonous plants (dicots). However, this promoter has lower activity in monocotyledonous plants (monocots), so alternatives such as the maize ubiquitine promoter are preferred[9].

One of the most important factors in governing the yields of recombinant proteins is subcellular targeting, which affects the interlinked processes of folding, assembly and post-translational modification of the protein. It has, for example, been shown in comparative experiments with recombinant antibodies that the secretory pathway is a more suitable environment for folding and assembly than the cytosol[10]. Proteins are targeted to the secretory pathway through the inclusion of an N-terminal signal peptide in the expression construct. In the absence of targeting information, proteins in the endomembrane system are secreted into the apoplast. The apoplast is the extracellular space, which is a large and continuous network of cavities under the cell wall. Proteins secreted from the cell often remain trapped here. However, yields are generally higher compared with secretion[11].

Even when carefully designed, transgene expression is influenced by several factors that cannot be controlled precisely through construct design. This leads to variable transgene expression and, in some cases, to its complete inactivation. Such factors include the position of the transgene integration, the structure of the transgenic locus, gene-copy number and the presence of truncated or rearranged transgene copies. Several strategies have been adopted in an attempt to minimize variation in transgene expression, including the use of viral silencing suppressors[12]. Currently the minimization of positioning effects remains an active field of research. Researchers are trying to establish methods by which a single-copy transgene can be integrated into a precise location in the plant nucleus[13]. In practice, however, commercially developed transgenic plants undergo an enormous amount of screening to identify phenotypic, yield and agronomic variations.

2.2.1.2 Post-translational modifications

The possibility of plant-specific glycans inducing allergic responses in humans has been considered and the finding that human serum contains antibodies that are reactive against these residues has been interpreted as evidence[14]. In addition it has been shown that the ß(1,3)fucose and ß(1,2)

---

8 Irniger *et al.* 1992.
9 Christensen and Quai 1996.
10 Trombetta and Parodi 2003.
11 Twyman *et al.* 2003.
12 Brignetil *et al.* 1998.
13 Butaye *et al.* 2005.
14 Gomorda *et al.* 2005.

xylose residues lead to adverse reactions[15]. However, in general, carbohydrate epitopes are rarely allergenic. Currently it is too early to generalize about how crucial humanized glycolysation of plant-derived pharmaceuticals is, and whether it might be more important to some classes of proteins than to others. Similarly, the method of administration (oral versus injection) could make a difference in terms of the immune response that might occur.

Plants are the production platform of choice in cases where innate mammalian molecules could interfere with the drug. For example, plants are being used as a production platform for the vitamin-binding recombinant human intrinsic factor (rhIF)[16]. Since plants do not use vitamin B12, contain vitamin B12, or have any proteins with affinity for vitamin B12, they can serve as a source for the vitamin-binding recombinant human intrinsic factor that is free from any vitamin B12 binding interferences. Porcine gastric-derived intrinsic factor preparations, in contrast, are often contaminated with haptocorrin, a vitamin B12 binding interference[17]. Another important reason to utilize plants as a production platform is that they are free of pathogens or prions that might be harmful to humans[18]. In addition, in cases where biopharmaceutical has to be stored or serve as an edible drug plants are the appropriate production platform[19].

2.2.1.3 Plant transformation method

Two general methods are used to generate transgenic plant lines for pharming: *Agrobacterium*-mediated transformation[20] and particle bombardment, in which DNA-coated microprojectiles are shot into plant tissue[21]. Each method has advantages and disadvantages, and the choice depends on a combination of factors, including selected host species, local expertise and intellectual property issues. Figure 2.3 illustrates the steps to be taken to achieve plant transformation with each method.

Agrobacterium mediated transformation (illustrated in table 2.2) makes use of a naturally occurring pathogenic soil bacterium, which has the ability to transfer parts of its own DNA into plant cells. In the wild, transfer of a portion of the bacterial DNA (called T-DNA, for "transfer DNA") causes rapid plant cell division leading to the formation of a tumour. Scientists have taken advantage of this naturally occurring transfer mechanism, and designed DNA vectors from the tumour-inducing plasmid DNA (*ti*-plasmid) found in the bacteria that is capable of carrying desired genes

---

15 Bardor *et al.* 2003.
16 Pujol *et al.* 2007.
17 www.cobento.dk (July 2008).
18 Giddings *et al.* 2000.
19 Mason *et al.* 1992.
20 Schlappi and Hohn 1992.
21 Klein *et al.* 1992.

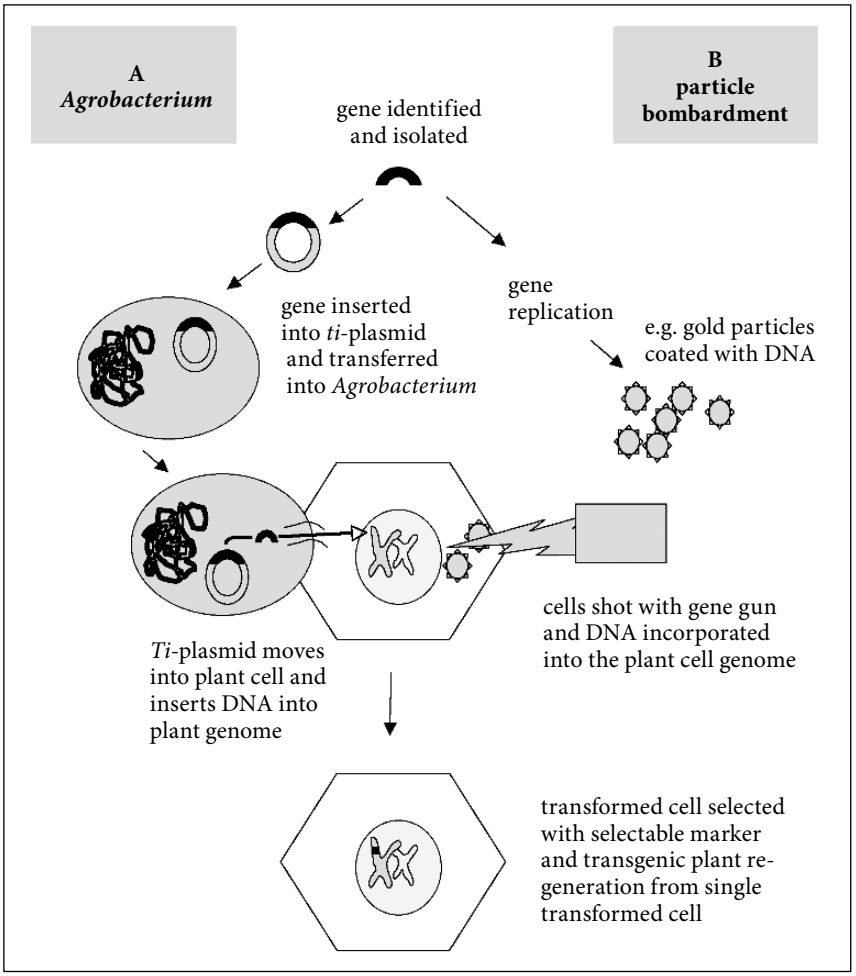
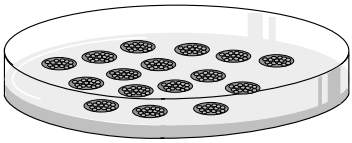
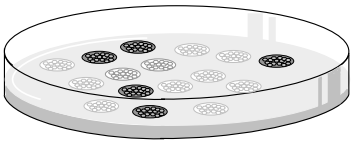
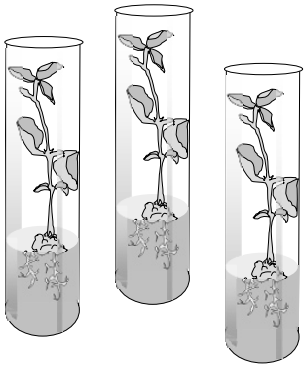


Figure 2.3: Strategies for genetic engineering of plants

into the plant. The engineered or constructed genes are inserted into the *Agrobacterium* vectors and enter the plant by the bacteria's own internal transfer mechanisms. For *Agrobacterium* to transfer part of its DNA into plants, living, wounded plant tissue is usually inoculated with the bacterium. After culturing the bacteria with the plant tissues, antibiotics are supplied, eliminating the bacterium from the plant tissue. The transformed plant tissue is then regenerated into a mature plant through tissue culture techniques[22].

[22] Kumria *et al.* 2001.

Table 2.2: *Agrobacterium* mediated transformation of plants

	For the transfer of foreign genes, leaf pieces are incubated with the transformed *Agrobacterium tumefasciens* strain.
	Leaf discs after transformation with Agrobacterium. For re-growth they are then cultivated on selective medium giving rise only to those cells which have been genetically transformed by the Agrobacteria.
	The transformed plant tissues can be regenerated to intact plants. Regenerated transformed tobacco plants grown *in-vitro*.

Particle bombardment, also referred to as the biolistic system, is a physical method for DNA delivery (illustrated in table 2.3). For this method, DNA is coated onto small (<1 μm), for example gold particles, which are accelerated towards the target plant tissues. The tissue could for example be a plant callus or leave discs. A plant cell callus consists of somatic undifferentiated cells from an adult plant that has the ability to differentiate. The bombardment devices use a sudden release of compressed helium gas to accelerate the DNA-coated particles. After the particles pass through the plant cell wall, they enter the cytoplasm and preferably the nucleus, where the DNA comes off the particles and integrates into the genome. Depending on the host species, several physical and biological parameters have to be adapted to achieve transformation. Physical parameters include the nature, chemical, and physical properties of the particles; the nature, preparation,

and binding of DNA onto the particles; and the characteristics of the target tissue. Biological factors include choice and nature of explants, pre-and post-bombardment culture conditions, and interactions between the introduced DNA and cytoplasmic or nuclear components. After the bombardment transformed cells have to be screened. Based on the number of bombarded explants, the overall transformation frequency can be for example as high as 15 percent with germline transformation frequencies approximating 0.25 percent[23]. The screening and testing for successful integration and expression of new DNA is performed by molecular techniques, such as a using tandem selectable gene markers or molecular analysis methods such as northern blots. Selected single cells from the callus or leave disc can be treated with a series of plant hormones, such as auxins and gibberellins, which are capable to induce the re-differentiation into entire plants. This capability of total re-generation is called totipotency. The new plant that originated from a successfully shot cell may have new genetic (heritable) traits. Their ability to segregate in a Mendelian fashion in the next generations is tested in the next step. Then the families that express the transgene in the desired manner are screened.

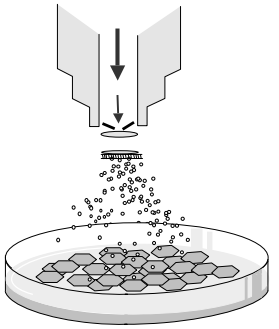
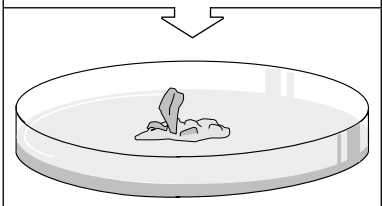
The choice of the transformation method depends largely on the plant species that is intended to be transformed. The soil pathogen *Agrobacterium tumefaciens* provides a simple method of transforming most dicotyledon[24] plant species and is commonly used for pharming in tobacco, alfalfa, pea tomato and potato[25]. Some selected monocotyledons (i.e. grasses) can also be transformed by *Agrobacterium*, but in most monocotyledons particle bombardment is the preferred method. This is the case for cereals, such as rice, wheat, and maize, but also for soybean and other legumes[26]. Particle bombardment is also necessary, if the target tissues are plastids, as the *Agrobacterium* T-DNA complex is targeted to the nucleus and therefore unsuitable for gene transfer to chloroplasts.

These transformation methods generally lead to the introduction of surplus DNA sequences into the genome of the host. In the case of *Agrobacterium*-mediated transformation, this is because inefficient processing of the T-DNA border sequences often results in cotransfer of flanking sequences[27]. In the case of particle bombardment, superfluous DNA transfer occurs because whole plasmids are generally used to coat microprojectiles[28]. Superfluous DNA transfer is a safety as well as a regulatory issue, when the result-

---

[23] Sanford *et al.* 1987; Sanford 1988.
[24] Dicotyledons, or "dicots" are a flowering plants whose seed contains two embryonic leaves or cotyledons. Monocotyledons – in contrast – typically are having one embryonic leaf.
[25] Giddings *et al.* 2000.
[26] Ma *et al.* 2003.
[27] Gelvin 2003.
[28] Klein *et al.* 1992.

Table 2.3: Particle bombardment mediated transformation of plants

	Gene Gun: When the fire switch on the outside of the chamber, helium is released at high pressure. The blast ruptures a first disk and a shock wave is induced. This wave hits another disk, which is free to move. Attached to the front of that disk are particles 1 micron in diameter coated with DNA molecules. This disk travels another centimeter at the speed of a gun projectile, roughly 400 meter per second, and hits a screen which detains the disk but liberates the DNA coated particles toward the target cells.
	Transformed cells are selected with selectable marker or by screening for the transgene with molecular methods. Selected single cells form a plant cell callus.
	Transgenic plant regeneration from plant cell callus and analysis of the transgenic plant lines.

ing genetically modified organisms (GMO) are to be released into the environment. Recently, several strategies to avoid the cotransfer of flanking sequences have been developed. One example is the incorporation of the *barnase* gene in the construct of *Agrobacterium* mediated transformation[29]. This gene ensures that all plant cells that contain sequences linked to the T-DNA are killed, as *barnase* gene expression is lethal. The *barnase* gene is derived from the soil bacterium *Bacillus amyloliquefaciens* and codes for a ribonuclease. In cases where flanking regions have undeliberatly incorporated into the genome of the plant, the *barnase* ribonuclease is synthesized. After activation the ribonuclease destroys the vital RNA molecules of the plant cell and is therefore lethal. By the addition of the *barnase* gene

[29] Beals and Goldberg 1997; Kuvshinov *et al.* 2004.

in the flanking regions only plant cells survive that have not incorporated these additional pieces of DNA. One side effect of these new methods is that the transgenic loci are considerably simpler than those of whole-plasmid transformants, and the plants show a notable reduction in the frequency of transgene silencing.

2.2.2 Transient expression using viral vectors

In addition to the incorporation of a transgene into the plants genome, a foreign gene can also be expressed transiently. That means that the transgene has not been physically incorporated into the genome but is carried as an episome that can be lost. In pharming genetically modified plant viruses have been applied to whole-plant systems for the production of transient biopharmaceutical expression[30]. In these cases the plant itself is not transformed with the gene for the biopharmaceutical, but a plant virus that uses the plant to propagate. This means that expression levels will not be constant over time, and will eventually fall away. It also means that the plant does not inherit the ability to produce the biopharmaceutical but the plant virus. Foreign proteins produced using viral vectors can be in the form of free cytosolic proteins or fusions to viral proteins. Viral expression systems exploit the ability of viruses to propagate rapidly and achieve high concentrations in plant tissues. For example, tobacco mosaic virus (TMV) can accumulate in infected tobacco leaves to levels greater than 60 mg/g dry weight and produce amounts of TMV coat protein accounting for 10–40 % of the total protein content of the leaves[31]. Provided the movement proteins on recombinant viruses remain functional, viral vectors are able to spread throughout the entire plant from a single infection point via the plasmodesmata between individual cells and the vascular system. Therefore, in principle, when foreign protein is co-expressed with the plant virus, large amounts of product can be found. However, currently the application of transgenic viruses in whole plants has not resulted in the production of foreign proteins to the same high levels as the viral proteins from non transgenic virus infections. This is probably because the genetic construct carried by the virus interferes to some extent with the normal folding, packaging, transmission and replication process. Nevertheless, foreign protein yields achieved using viral vectors can be substantial. For example, transgenic viruses with coat protein fusions have been reported to accumulate to levels of 1–3 mg/g plant tissue[32].

---

30 Grill *et al.* 2005.
31 Shadwick and Doran 2007.
32 Shadwick and Doran 2004.

2.2.3 Choice of species and site of production

The choice of the expression platforms in plant pharming depends on a combination of factors, including environmental conditions of the intended growing area, local expertise and intellectual property issues. In addition factors that must be considered when choosing a production crop include biomass yield per hectare, yield of the recombinant protein per unit biomass, ease of transformation and scalability. Also it needs to be taken into account whether the biopharmaceutical is intended to be consumed together with the plant (for example edible vaccines) or whether it should be stored before isolation (for example in the grain) whether the biopharmaceutical is intended to be isolated immediately after harvest (for example from leafs). The following expression organs can be differentiated: leafs, seeds and fruits or vegetables. In addition to the utilization of whole plants, fermenter grown plant cell suspensions or root cultures are applied in plant pharming.

2.2.3.1 Leaves

Tobacco *(Nicotiana tabacum)* has an established history as a model system for pharming and is the most widely used species for the production of recombinant pharmaceutical proteins at the research laboratory level[33]. It is therefore one of the strongest candidates for the commercial production of recombinant proteins. The major advantage of tobacco includes the well-established technology for gene transfer and expression, high biomass yield, prolific seed production and the existence of a large-scale processing infrastructure. Because tobacco is neither a food nor a feed crop, there is little risk that tobacco material will contaminate either the food or the feed chain. Although many tobacco cultivars produce high levels of toxic alkaloids, low-alkaloid varieties are available that can be used for the production of pharmaceutical proteins[34].

Alternative leafy crops that are being investigated for pharming include alfalfa, soybean and lettuce. Alfalfa and soybean have the major advantage of using atmospheric nitrogen through symbiotic nitrogen fixation, which therefore reduces the need for chemical fertilizer. Alfalfa is in particular useful because it has a large dry biomass yield per hectare and can be harvested up to nine times a year. Both of these legumes have been used to produce recombinant antibodies. Lettuce is also being investigated as a production host for edible recombinant vaccines and has been used in one series of clinical trials for a vaccine against hepatitis B virus[35].

One great disadvantage of leafy crops is that recombinant proteins are synthesized in an aqueous environment and are often unstable, resulting in low yields. The leaves must be frozen or dried for transport, or processed soon after harvest to extract useful amounts of the product.

---

[33] Richter *et al.* 2000.
[34] Ma *et al.* 2003.
[35] Twyman *et al.* 2003.

2.2.3.2 Cereals, legume seeds and oilseeds

Numerous crops have been investigated for seed-based production, including the cereals rice, wheat oilseed rape and maize; the legumes pea and soybean[36] and the oilseed sallower[37]. The advantage of the expression of proteins in seeds over leafy crops is the possibility to long-term storage, even at room temperature, because seeds have the appropriate biochemical environment to promote stable protein accumulation. Cereal seeds also lack the phenolic compounds present in tobacco leaves, thus improving the efficiency of downstream processing. However the overall yields of recombinant proteins in seed crops are much lower than in tobacco, and the most appropriate system must be determined on a case-by-case basis.

2.2.3.3 Fruits and vegetables

A major aim of protein expression in fruit and vegetable crops is that the edible organs can be consumed as uncooked, unprocessed or partially-processed material, making them suitable for the production of recombinant vaccines and antibodies designed for topical applications. Potatoes are the major system for vaccine production. Tomatoes are more palatable than potatoes and have other advantages including high biomass yield and the use of greenhouses increases containment[38].

One of the main aims to develop edible vaccines is the fact that they could be distributed without refrigeration, which could be of importance – especially in developing countries. Bananas are feasible vehicles for edible vaccine distribution – but to date problems of inconsistent expression levels in the fruits due to different growth conditions and the resulting inconsistent doses are not yet solved[39].

2.2.3.4 Plant cell cultures and hairy root systems

In some cases fermenter grown large-scale plant cell cultures or hairy root cultures might offer an alternative route for recombinant protein production[40]. These production platforms can be chosen in situations when the production of biopharmaceuticals in field grown plants comes along with too many obstacles: In cases where the foreign protein is toxic, for example to soil microorganisms or to wild-life capable of consuming the plants, issues of environmental safety might be too hard to solve. In addition agricultural production of delicate proteins might fail to provide adequate assurance of product safety and quality, for example when weather and soil

---

[36] Burkhardt *et al.* 1997; Stöger *et al.* 2000; Azzoni *et al.* 2002.
[37] Cory *et al.* 2006.
[38] Conrad and Hain 2005.
[39] Lal *et al.* 2007.
[40] Shadwick and Doran 2007.

conditions are hard to control or the fields are subject to contamination with pesticides, herbicides and mycotoxins.

Even when a very important advantage of agricultural plant pharming – the cheap and faster scale up procedure – is lost in this production platform, plant tissue cultures and root cultures still offer a number of advantages over currently utilized mammalian cell cultures: One important factor is that plant cell culture media are relatively simple in composition and less susceptible to contamination with undesirable organisms, which leads to reduced material and process costs. Another advantage is that plant culture media do not contain proteins – thus the recovery of the desired protein is easier and cheaper than mammalian cell cultures. In addition as most plant pathogens are unable to infect humans or animals, the risk of pathogenic infection being transferred from cell culture via the product is minimized.

Plant cell cultures comprise small aggregates of undifferentiated plant cells in liquid nutrient medium. Dedifferentiation and the promotion of growth are achieved by the addition of plant hormones to the medium. Tobacco has been the species most studied but rice *(Oryza sativa)* has also been utilized by several groups[41]. The predominance of tobacco in plant cell cultures differ from the situation of transgene expression in whole plants, where advantages associated with producing the pharmaceutical protein in edible species or in different storage organs such as seeds have resulted in a variety of species being transformed (see table 2.1). Obstacles in plant cell culture are that they are often subject to genetic instability that might cause significant reduction in yield of the foreign protein over time and that to date few plant cell cultures have shown to accumulate or secrete biopharmaceuticals at concentrations sufficient for commercial viability[42].

Hairy root cultures are a second system for protein production in culture (illustrated in table 2.4). They comprise roots that can grow independently of the plant in liquid nutrient medium due to action of phytohormones. They are called "hairy" because their morphology is more branched and thinner than roots adherent to the plants. In contrast to plant cell cultures exogenous plant hormones are not applied to the medium, but are synthesized by the roots themselves after they have been transformed with the bacterium *Agrobacterium rhizogenes*. These bacteria transmit the genes that code for the phytohormones to the host plant that afterwards produce hairy roots. Biopharmaceutical expressing hairy roots can be obtained by the infection of transgenic plants with *Agrobacterium rhizogenes* or can be produced by performing root initiation and transformation with the recombinant gene at the same time using genetically modified *Agrobacterium rhizogenes* with the transgene inserted into the plasmid construct, that is as described transmitted to the host plant. When cultured in liquid medium, hairy roots often

41 Kim *et al.* 2008; Lee *et al.* 2007; Jung *et al.* 2006.
42 Shadwick and Doran 2004.

Table 2.4: Hairy root cultures: establishing and manufacturing

	Hairy root formation, after infection with *Agrobacterium rhizogenes*. The plant pathogen induces tumour-like hairy root formation, by transforming the plant with genes for tumour inducing plant hormones. Transformed roots are selected by the addition of antibiotics, to liberate the roots from the plant pathogen. They therefore become sterile and are able to grow in liquid media.
	After the transformation of the hairy roots with the desired gene for biopharmaceutical expression, the hairy root cultures are scaled up for the growth in production vessels.

Source (pictures): ROOTec, Basel

exhibit rapid growth relative to untransformed roots, due to plant hormones. They can be propagated indefinitely in liquid medium and have been found to have significantly greater long-term stability than plant cell cultures.

2.2.4 Cultivation

While plant cell cultures or hairy root systems are propagated in contained environments (vessels) the large-scale cultivation of transgenic plants is carried out in greenhouses or, for reasons of economy and scalability, in the field. A series of varying environmental factors must be taken into account at this step. They include light intensity, temperature, water regime, soil quality, the kind of fertilization, the presence of pests and the substances utilized to treat them. These factors are important on the level of the quantity of the yield but also on the level of its quality (See section 2.4). For containment strategies see chapter 3.

2.2.5 Purification of biopharmaceuticals from transgenic plants

2.2.5.1 Purification of biopharmaceuticals from whole plants

Besides the level of expression of a recombinant protein the subsequent purification steps are crucial for the resulting yields. The process includes the initial processing of the source material (i.e. grinding of the grain), extraction, capture, intermediate purification and polishing (see also figure 2.2). The

key issues in these steps are removal of host cell proteins and nucleic acids, as well as other product or process related or adventitious contaminants[43].

After harvesting, leaves and other soft tissue begin to degrade. Therefore the conditions and duration of storage before initial processing are critical. In seed based production systems this step is less important, since long-term storage is possible. Therefore in seed based systems it is possible to separate the place and time of harvesting and the initial processing of the material. Once the material is harvested and introduced to further processing, all further steps must be performed according to the regulations of pharmaceutical GMP (good manufacturing practice). That includes the need for equipment qualification and process validation. Since the expression level and activity will depend on the environmental conditions and plant health for field-grown plants a validation of the process will include the definition and separation of batches. The maintenance of a batch to batch consistency can not always be achieved fully. The situation is different for plant cell cultures cultivated in bioreactors, since growth conditions are controlled in this case and batch variance can therefore be minimized.

The initial processing of the plant material is routine, because machinery, for example corn mills, leaf shredders etc., are already available from suppliers from food and feed industry. The design of the initial processing step is largely dependent on the source material. The most commonly used target tissues for the expression of recombinant proteins are leaves and seeds. Leaf material contains a lot of water. For the extraction of the desired protein from leaf material it can be homogenized and extracted in its own juice. The addition of extraction buffer is mainly needed to control pH and to introduce protein-stabilising agents. Seeds, on the other hand, must be subjected to dry milling followed by extraction in a large proportion of buffer. Before purifying the target protein from the crude plant extract, a clarification step is required to separate particulate material from liquid phase. In the case of leaves as row material, leaves are disrupted by shredding. Therefore fibres and other fine materials are inevitably generated. Even after removal of bulk cell for example by centrifugation or material depth filtration a portion of fine green material will remain that could in the following chromatographic purification block the columns. Thus an additional step of cross-flow microfiltration has to be incorporated in the purification process.

After extraction the following steps of capture, intermediate purification and polishing of the target protein are basically performed by chromatographic purification. All supplementary procedures like extraction, centrifugation and filtration ultimately serve to condition the protein for chromatography. A series of chromatographic steps, usually termed capture, intermediate purification and polishing, making use of different intrinsic features of proteins, is usually required to archive sufficient separation

[43] Drossard 2004.

of the target from contaminants[44]. Common modes of chromatography include ion exchange chromatography, affinity chromatography, hydrophobic interaction chromatography, gel filtration and to a limited extend, reverse phase chromatography[45].

The whole extraction process contributes significantly to the overall costs of the production of biopharmaceuticals. A majority of product-specific requirements for the purification from the raw material are not associated with the particular expression system, leading to the conclusion that potential economic advantages of plant production systems lie in the upstream rather than the downstream (purification) part of the process. The large contribution of the downstream costs to the overall cost of the process will always put pressure on the this side of the process

In the whole process of purification, expression specific risks for product quality and safety must be adequately taken into account. In contrast to established host systems like *Escherichia coli* and mammalian cells, the regulatory requirements for plant based pharmaceuticals are not fully defined. However, both the US Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products (EMEA) have recently published draft guidance documents addressing this issue[46].

2.2.5.2 Purification of biopharmaceuticals from plant cell cultures and hairy root cultures

Purification of biopharmaceuticals from plant cell cultures follows the same pattern as the purification of recombinant proteins from field grown plants: initial processing of the source material, extraction, capture, intermediate purification and polishing. However often in cell cultures many of these steps can be circumvented by an ingenious molecular strategy: Proteins produced in plant cells can remain within the cell or are secreted into the apoplast via the secretory pathway. Foreign proteins are generally not directed for secretion but can be added with a small piece of signal sequence that initiates their secretion. In plant cell cultures an excretion of the recombinant protein to the culture medium can be achieved by this strategy[47]. The biopharmaceutical can than be harvested directly from the culture medium and purification to remove the host cell proteins and nucleic acids is not required. Thus protein purification from whole plant biomass is potentially much more difficult and expensive than protein recovery from culture medium and protein secretion is considered an advantage in plant cell culture systems. However not all biopharmaceuticals are suitable for protein excretion since i.e. their stability after secretion in the culture medium can not always be ensured. Also the size of the protein might be crucial: Secre-

---

44 Fahrner *et al.* 2001.
45 Further reading: Drossard 2004.
46 FDA 2002; EMEA 2002.
47 Sharp and Doran 2001.

tion of recombinant proteins into the medium requires that they pass pores in the cell wall of the plant cell. The pores in plant cell walls are thought to allow passage of proteins of maximum size around 20 kDa. However a small number of wider pores may serve as channels for relatively slow secretion of larger molecules. Also protein composition and structure may affect the extracellular availability of recombinant protein: in many expression systems it has been described that despite an appropriate signal sequence an considerable amount of foreign protein remain within the plant cell or remain associated with the plant cell wall[48]. Depending on the recombinant protein plant cell cultures and hairy root cultures can also differ in their ability to secrete the recombinant protein, which could mean that the one or the other system is the preferable choice.

2.3 Animals as a production platform for recombinant biopharmaceuticals

Animal pharming involves the expression of protein products in whole animals, principally livestock. Production in mammalian cell culture is not included under this definition. This section provides an outline of basic recombinant DNA technology used in animal pharming, reviews methods of generating transgenic animals, describes technical issues affecting the choice of species and tissue used for production, and briefly describes purification of protein products from transgenic animals.

2.3.1 Transgene constructs used for animal pharming

In animal pharming, the basic intentions underlying the design of all transgene constructs are that the construct should integrate into the host genome, be inherited in classic Mendelian fashion and direct the abundant expression and secretion of a desired protein without affecting the health and well-being of the producing animal.

The first step in expressing any protein in a transgenic animal is to obtain a region of cloned DNA that encodes the amino acid sequence. This may be a cloned fragment of genomic DNA, complementary DNA (cDNA) or chemically synthesized DNA. In some cases, it may be necessary to alter this coding sequence to make it suitable for expression in a transgenic animal. For example, if the protein is from an evolutionarily distant species, it may be necessary to alter codons to accord with those most frequently used in the host. Also, if the protein is not normally secreted, a signal peptide may be added at the N-terminal to direct secretion from the producing cell, for example into milk.

Furthermore, one needs a gene that is expressed specifically and preferably abundantly in the tissue to be used as the site of production. For example, for the lactating mammary gland milk genes such as beta-casein or

[48] Matsumoto *et al.* 1995.

beta-lactoglobin would be appropriate, or for production in chicken eggs the ovalbumin gene. These genes provide control elements that can be used to confer tissue-specific expression on foreign gene sequences. Typically, these are the gene promoter, where gene transcription initiates, one or more enhancers that positively regulate transcription activity, and inhibitory elements with negative influence. Animal pharming has been heavily reliant on genes whose regulation has been well studied; genetic control elements can sometimes be distant, dispersed and ill-defined.

Many researchers have found that transgenes based on genomic sequences are expressed more consistently and more abundantly than those based on cDNA. However, many genes are too large to be conveniently inserted into bacterial plasmid cloning vectors. As a compromise, cDNA and genomic sequences may be combined in a "minigene" carrying one or two introns, and 5' or 3' flanking regions known, or suspected, to contain regulatory elements. It has, however, been found that mixing and matching in this way can sometimes lead to aberrant RNA splicing due to the presence of cryptic splice sites. Such effects are often difficult to predict in advance.

Despite considerable knowledge of the control of gene expression, there is at present no standardized method of combining disparate genetic elements that is guaranteed to achieve successful transgene expression. Transgene design is a matter of informed trial and error, usually requiring test and refinement of successive generations of constructs.

An important factor determining the success of transgene expression lies not in the construct itself, but in the location at which it integrates into the host genome. Transgenes integrated at different sites can exhibit wide variations in expression, due to the influence of different chromatin environments. Heterochromatic regions tend to suppress expression of an adjacent transgene, while a transcriptionally active chromatin domain may support expression. The proximity of endogenous enhancers, promoters, silencers and activation sequences may also influence the level and pattern of transgene expression. This "position effect" has been a long-standing source of inefficiency in the use of transgenic animals. A review[49] provides further details of the effect.

Several strategies are available to either minimize or circumvent the position effect. Improvements in the level, specificity and consistency of transgene expression can be gained by flanking the transgene construct with insulator elements. Insulators of various types have been demonstrated to block the spread of heterochromatin into the transgene, and isolate transgenic promoters from the effects of adjacent endogenous enhancers and other regulatory elements. Alternatively, the uncertainties of random integration can be avoided by placing the transgene construct at a well-characterized permissive site in the host genome using gene targeting, as described

[49] De Laat and Grosveld 2003.

in section 2.3.2.4. The Rosa26 locus in the mouse is one example of a commonly used transgene locus that is not subject to gene silencing. Placing recognition sites for either a recombinase, for example bacteriophage P1 Cre recombinase, or an integrase, for example phage ϕC31 integrase, at the locus allows transgene cassettes to be inserted at very high efficiency by so-called recombinase-mediated cassette exchange[50]. A human homologue to the mouse Rosa26 locus has recently been identified[51] and it is probable that similar sites will also be identified in livestock.

A quite different approach is to avoid transgene integration altogether. The problem, then, is to ensure that the foreign DNA replicates autonomously and is stably maintained after multiple cell divisions. This can be achieved using so-called artificial chromosome vectors that carry DNA regions responsible for stable chromosome behaviour. These are: a centromere that mediates chromosome segregation during mitosis, and two telomeres that stabilize the ends of the DNA molecule. Artificial chromosome vectors have the advantage that they can carry very large regions of DNA; however, they are still in early stage development. There has been one report of "transchromosomic" cattle, designed to express human immunoglobulins in blood[52].

A second form of non-integrating, or episomal, vector has been developed that incorporates an attachment site for the system of structural proteins within the nucleus, termed the nuclear matrix or scaffold[53]. Such vectors are thought to achieve mitotic stability by "piggybacking" on chromosomes. Episomal vectors are not widely used for generating transgenic livestock, but there has been one report of the production of pigs expressing a fluorescent reporter gene[54].

2.3.2 Methods of producing transgenic livestock

Mammalian oocytes and early stage embryos are tiny, self-contained, free-floating structures that can be obtained relatively easily by flushing the female reproductive tract with fluid. They can be kept in culture and then reintroduced into recipient females to continue gestation. This accessibility has facilitated the development of a variety of micromanipulation, culture and embryo transfer procedures which support the production of transgenic mammals. Techniques for avian transgenesis are less well-developed. Early stage avian embryos are attached to a large fragile yolk and, once explanted, cannot easily be transferred back into the oviduct, necessitating *ex vivo* embryo culture[55].

---

[50] Hitz *et al.* 2007.
[51] Irion *et al.* 2007.
[52] Kuroiwa *et al.* 2002.
[53] Jenke *et al.* 2004.
[54] Manzini *et al.* 2006.
[55] Love *et al.* 1994.

The first viable transgenic mammal was produced in 1974 at the University of California, San Diego, when Rudolf Jaenisch and Beatrice Mintz injected simian virus 40 particles into the cavity of a mouse blastocyst-stage embryo. Two years later, Jaenisch reported that mouse embryos could be infected with a retrovirus which stably integrated the proviral DNA into their genome. These animals incorporated the retroviral DNA into their somatic tissues and germ line and passed it on to their progeny in Mendelian fashion. Since then, transgenic animals have proved to be valuable research tools and are gradually finding practical applications, for example as models of human disease, in xenotransplantation and animal pharming. The term 'transgenic' is now generally used not only to describe animals bearing additional exogenous DNA, such as an expression construct, but also those carrying other forms of engineered genetic change, such as gene replacement, deletion, inactivation or alteration. In this section, we provide an overview of various methods of generating transgenic mammals and birds, including those in routine use and some still being developed.

The methods divide into two broad categories: nucleic acid transfer directly into embryos (sections 2.3.2.1 to 2.3.2.3) and cell-mediated transgenesis (sections 2.3.2.4 to 2.3.2.8). Nucleic acid transfer into embryos is more straightforward than cell-mediated transgenesis. However, it is at present limited to transgene addition and allows no control over where the transgene integrates into the genome. Definitive analysis of the integrated transgene must be carried out in resultant animals, which in livestock is costly and restricts the number of independent integrations that can feasibly be generated and investigated. The various methods of cell-mediated transgenesis each have in common the feature that genetic manipulation and analysis of the transgenic genotype is carried out in cells in the laboratory, rather than in animals "on the farm". These cells are then used to transfer the modified genotype to whole animals. While cell-mediated transgenesis is more labour intensive than direct transgenesis, *in vitro* genetic manipulation of cells followed by detailed genome analysis offers significant advantages. Firstly, it reduces the total number of animals required to generate a useful transgenic. Secondly, it increases dramatically the number of independent transgene integration events that can be screened and investigated. Thirdly, it facilitates the engineering of precisely controlled genetic alterations (gene targeting) by allowing selection and isolation of rare integration events resulting from homologous recombination (see section 2.3.2.4).

2.3.2.1 Pronuclear DNA microinjection

In 1980, Frank Ruddle of Yale University reported that naked DNA, mechanically microinjected into the pronuclei of fertilized mouse eggs, could stably integrate into the host genome[56]. Microinjection by the same basic pro-

[56] Gordon *et al.* 1980.

cedure was successfully extended to livestock shortly after[57]. DNA microinjection is rarely used in birds, but in mammals it offers a straightforward method that has been used to produce transgenic pigs, cattle, rabbits, goats and sheep. A wide variety of transgenes have been microinjected, including DNA fragments as large as 0.5 mb and combinations of up to four different transgenes in mice, and three in sheep. The procedure may be divided into a number of stages as summarized below. Several of these stages are common to other methods.

Collection of fertilized eggs. Oocytes fertilized *in vivo* can be collected from animals induced to ovulate by hormonal treatment or from spontaneous ovulation. Alternatively, oocytes can be withdrawn from follicles of ovaries collected from slaughterhouses; in the United States companies have been established to provide such oocytes by post. Embryos are then produced by *in vitro* maturation and fertilization (IVM/IVF), often termed *in vitro* production (IVP). IVP procedures are well established for cattle and have recently been developed for pigs. This technique avoids the need for donor animals and has become the method of choice in cattle and is increasingly being used in pigs. Using current techniques, IVP fertilized oocytes are slightly less viable than those derived *in vivo,* but this is offset by the greater numbers available.

Preparation of DNA. A recombinant DNA construct used to generate a transgenic animal will generally have two broad components, the transgene portion and the bacterial plasmid "backbone" which comprises sequences required for selection and DNA replication in bacteria, usually *Escherichia coli*. Preparation of a DNA construct for microinjection entails purifying linear fragments of the transgene portion away from the plasmid DNA backbone, residual bacterial material and chemical reagents. These procedures are well developed standard molecular biology practice. Exogenous DNA is toxic to early embryos, therefore the quality and concentration of microinjected DNA is critical for survival. Purified linear fragments are dissolved in microinjection buffer at a concentration between 1–6 ng/μl. Within this range, lower concentrations result in higher embryo viability but a lower proportion of transgenic embryos, higher concentrations produce more transgenic embryos but viability is reduced.

Injection of DNA. Morphologically normal fertilized oocytes with visible pronuclei are selected for microinjection. In some species, for example pig and cattle, oocytes are relatively opaque due to abundant lipid vesicles, but transparency can be improved by localising the lipids using gentle centrifugation. Microinjection is carried out on the stage of an inverted microscope, with the fertilized oocytes contained within microdrops into which are inserted a blunt-ended, glass holding pipette and a sharp, glass microin-

[57] Hammer *et al.* 1985.

jection needle containing the DNA solution. Movements of the pipettes are controlled by micromanipulation arms (see figure 2.4). Individual oocytes are held in place on the holding pipette by gentle suction and oriented so that one of the pronuclei is adjacent to the needle. The needle is inserted into one of the pronuclei and DNA solution injected (figure 2.4b). Injected oocytes can then be either immediately transferred to recipients, or cultured in vitro, sometimes as far as blastocyst stage, to reveal those that sustain further development.

Transfer and gestation in recipients. Viable embryos are selected for embryo transfer and introduced into the oviducts of hormonally primed (or in mice, pseudopregnant) recipients to complete gestation. Transgenic animals that develop from manipulated embryos are conventionally referred to as "founders", because they may be used to found genetically modified lines by conventional breeding.

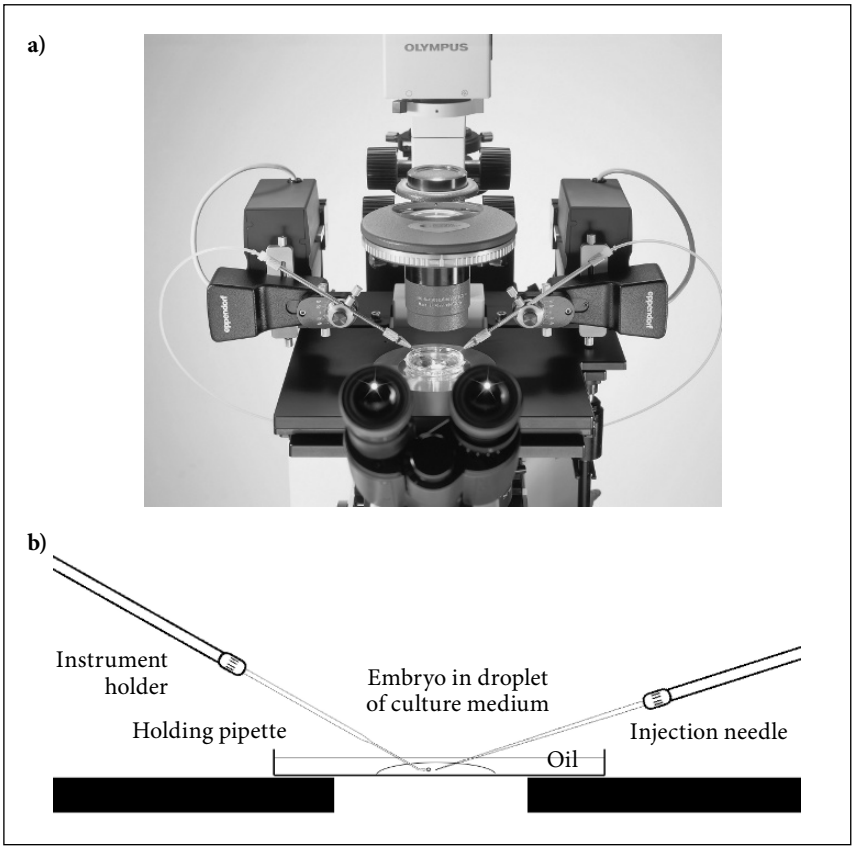


Figure 2.4: a) Typical micromanipulator stage (photographic image provided courtesy of Eppendorf)
b) Microinjection of a mammalian oocyte

Identification of founders and subsequent breeding. Microinjection is an inefficient process. Between 5–20 % of mice and 1–5 % of large animals born after microinjection carry a transgene, and a smaller proportion of these are likely to express the transgenic product. In mice, this inefficiency can readily be compensated for by injecting and transferring large numbers of embryos. However, this is frequently impractical in livestock because gestation times are longer and maintenance costs far higher. There has therefore been a strong incentive to reduce the number of animals gestating non-transgenic foetuses. One approach has been to screen embryos for the presence of a transgene before transfer. This can be achieved by extracting a portion of the embryo, often a single blastomere, and detecting the transgene by polymerase chain reaction (PCR) amplification. This procedure is however labour-intensive, can reduce embryo viability, and the presence of non-integrated DNA may result in false positives. Alternatively, a gene encoding a non-toxic fluorescent protein can be either co-injected, or incorporated into the transgene construct to identify intact living transgenic embryos. However, expression of a non-integrated reporter construct may again produce a false positive signal, and the presence of additional DNA may be undesirable.

Transgenic foetuses can be identified *in utero* by analysis of cells shed by the developing foetus obtained by amniocentesis or allantocentesis; however, these procedures carry a significant risk of inducing abortion. Some efforts have also been made to detect and analyse foetal cells or DNA in the maternal circulation, with limited success. The most common practice is therefore to screen animals shortly after birth by either PCR or Southern hybridization, using small samples taken from blood, tail or ear tips. Transgenic animals are then analysed in more detail to identify those most suitable for further breeding.

Where the intention is to express a protein, researchers will wish to determine the amount and properties of the protein expressed, and whether the physiology of the animal is affected in any way. Usually, some preliminary data on these parameters will already have been gained by mouse experiments before transgenic livestock are generated. However, prediction across quite distantly related species is necessarily imperfect. Furthermore, the position effect means that each founder is potentially different and must be analyzed independently.

Collection of milk expression data requires that female animals must be derived, attain sexual maturity, breed and lactate. This is necessarily time-consuming in livestock, especially where a founder is male. Protocols for artificial hormone-induced lactation in virgin females and even males have therefore been developed. This can accelerate the process of identifying the most suitable founders, but the quantity of milk obtained is often low, and expression data from induced lactation may differ from natural lactation.

Subsequent breeding from founder animals is carried out by conventional animal husbandry. However, it is not uncommon for founders produced by microinjection to be mosaic for the presence of the transgene. This is believed to be a consequence of delayed DNA integration. Mosaicism in the germ line reduces the frequency of transgene inheritance in the first generation. Mosaic components of a founder animal may possibly contain independent and different integrations of the transgene that segregate in the first generation. Transgene segregation due to founder mosaicism does not indicate transgene instability.

In summary, DNA microinjection is a straightforward method of generating transgenics, and has been the most commonly used over the past two decades. However, the limitations of random transgene addition and the inefficient use of animals are significant drawbacks, and radical improvements to the technique are not expected in the immediate future. We anticipate that it will soon be superseded by lentiviral transduction (section 2.3.2.2) and nuclear transfer (section 2.3.2.6).

2.3.2.2 Viral gene transfer

A number of viruses (for example SV40, AAV) have been used to generate transgenic animals with varying degrees of success. By far the most commonly used are retroviruses, see also review[58]. Retroviruses are a diverse group of viruses that share a basic common structure and replicative strategy. Each viral particle is approximately 100nm in diameter and consists of a protein "capsid" containing a genome of two single-stranded 8-11kb RNA molecules, surrounded by a lipid envelope through which glycoprotein spikes protrude. Retroviruses infect their hosts by binding the spike proteins to specific receptors on the cell surface. The virus enters the cell, converts the RNA genome into DNA and this integrates randomly into a host chromosome as a provirus. The transcriptional apparatus of the host cell is subverted to produce RNA from the provirus. Viral RNA transcripts include smaller species that encode viral protein components, and full-length genomic RNAs that are packaged into virus particles and bud off from the cell surface.

Retroviruses infect susceptible cells with very high efficiency and integrate as single copies into the host genome. This has led to the development of retroviral vectors capable of introducing foreign genes into cells, termed viral transduction. These vectors generally retain the outermost parts of the viral genome, termed long terminal repeats (LTRs) necessary for the production of genomic RNA, but lack viral genes and carry other disabling mutations. Retroviral vectors are therefore "replication incompetent", being unable to produce viral particles on their own.

---

[58] Buchschacher 2001.

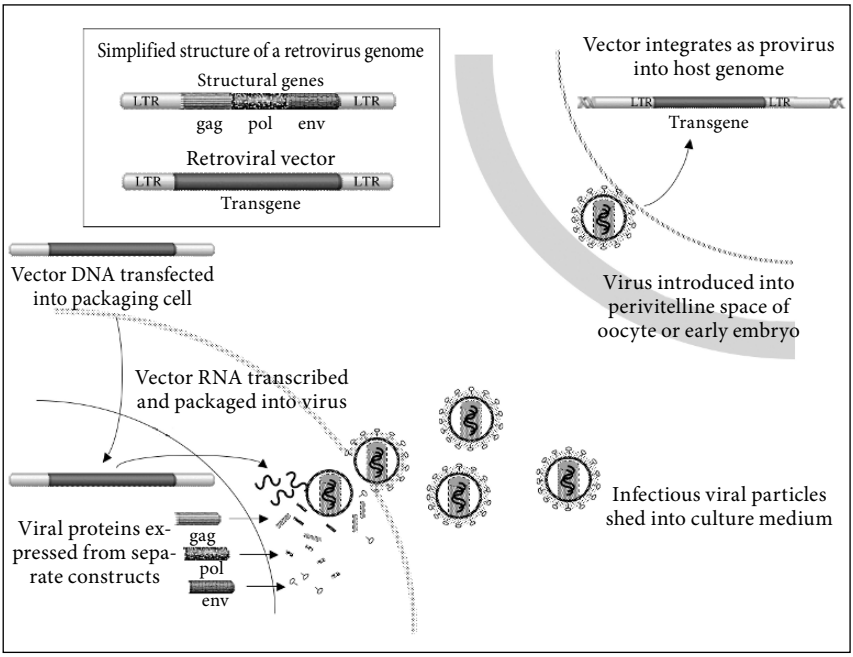


Figure 2.5: Transduction of a transgene using a replication-defective retroviral vector

Retroviral transgenesis is summarized in figure 2.5. The transgene is first inserted into retroviral vector DNA. This construct is transfected into an artificial "packaging cell line" designed to provide the viral components deleted from the vector. Typically, a packaging cell contains two or three stably transfected separate plasmids expressing viral genes: *env* which determines host cell specificity, *gag* encoding structural proteins and *pol* encoding the reverse transcriptase enzyme necessary to convert viral RNA into DNA.

The transfected vector generates genomic RNA transcripts within the packaging cell. These associate with the other viral proteins, become incorporated into infectious virus particles and shed into the culture medium. An embryo may be infected either by removing its *zona pellucida* and culturing in culture medium containing virus, or a small volume of viral supernatant may be microinjected into the perivitelline space. The virus vector can infect and incorporate into the host genome, but is incapable of further replication. Procedures for embryo explantation and transfer are basically those described for DNA microinjection.

Until recently, the majority of retroviral vectors were based on the oncoretrovirus Moloney murine leukemia virus, as used by Jaenisch. Transgenic mice, pigs and cattle have all been produced using these vectors, but rates

of transgenesis have been low and transgenes are almost always silenced by epigenetic modification during development. However, in 1996 scientists at the Salk Institute, San Diego, developed vectors based on lentiviruses. Lentiviruses are a subclass of retroviruses that include the sheep maedi-visna virus, equine infectious anaemia virus and the bovine, feline, simian and human immunodeficiency viruses. These offer two significant advantages over oncoretroviruses. Firstly, they are not subject to epigenetic inactivation in transgenic animals. This is probably because, unlike oncoretroviruses, lentiviruses are usually transmitted horizontally between individuals rather than vertically through the germ line. Host animals have, therefore, not adapted mechanisms to silence their expression. Secondly, unlike oncoretroviruses they do not require breakdown of the nuclear envelope to gain access to the host genome. Lentiviral integration can therefore occur at all stages from the unfertilized oocyte onwards, in both replicating and non-replicating cells. Lentiviruses have been used successfully in a number of mammalian species: mice, rat, pig and cattle. For further background see a review by Pfeifer[59].

Two publications, describing the production of pigs carrying a green fluorescent protein reporter transgene, illustrate the high rate of transgenesis obtained using lentivirus vectors. Alexander Pfeiffer and Eckhard Wolf of the Ludwig-Maximilians University, Munich used a vector based on human immunodeficiency virus and report 70 % of live born pigs carried a fluorescent protein transgene, of which 94 % expressed the transgene[60]. Bruce Whitelaw and colleagues of the Roslin Institute, Scotland obtained similarly high results in pigs using a vector based on equine infectious anaemia virus. 31 % of zygotes injected with virus developed to transgenic animals, of which 95 % expressed the transgene[61].

However, on the downside, delayed retroviral integration and independent integrations into different embryonic blastomeres are both quite common, and lead to a relatively high incidence of mosaicism in founders. This can extend the time required to establish stable transgenic lines; firstly, because mosaicism in the germ line reduces the frequency of transgene inheritance and secondly, because several generations may be required to segregate independent transgene loci.

Retrovirus-mediated gene transfer has been the principle means of producing transgenic chickens, and has also been used to produce transgenic quail. Infection is most commonly carried out on embryos within freshly laid eggs. At this stage the chick embryo comprises a multilayered plate, or blastoderm, of about 60,000 cells overlying the large yolk. The shell of the egg is removed and retroviral particles are injected into a fluid-filled space,

59 Pfeifer 2004.
60 Hofmann *et al.* 2003.
61 Whitelaw *et al.* 2004.

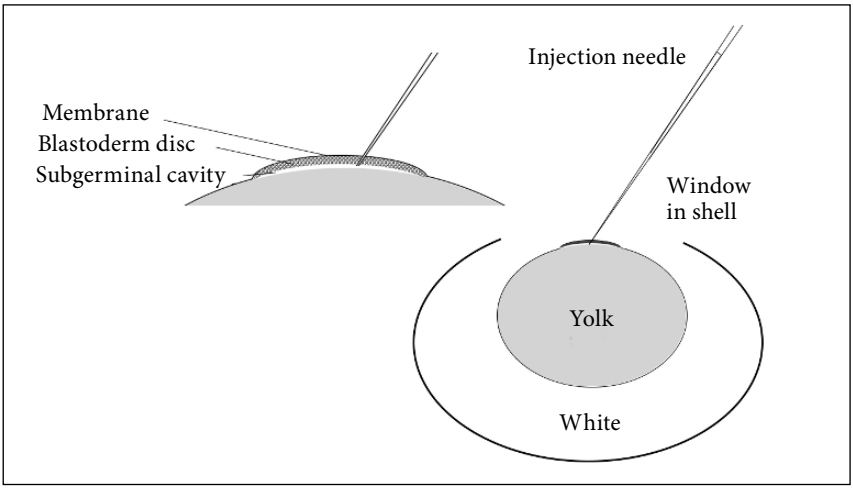


Figure 2.6: Injection of retrovirus particles into a hen's egg (shown in cross section)

the subgerminal cavity, that lies beneath the embryo, see figure 2.6. The shell is then resealed and the embryo allowed to incubate, sometimes being transferred to a surrogate shell after two or three days incubation.

Because infection takes place at a relatively late stage of development, founder birds are mosaic for retroviral integrations and must be bred on to segregate different proviruses. Early work used replication-defective vectors based on oncoretroviruses such as avian leucosis virus, or reticuloendotheliosis virus. The production of pharmaceutical proteins in bird eggs was first demonstrated using an avian leukosis virus vector to transduce a non-specific promoter directing the expression of interferon α-2b[62]. This resulted in extremely low frequencies of germ line integration and transgenes were frequently silenced over generations. More recent work using lentiviral vectors based on equine infectious anaemia virus has been more successful, and pharmaceutically relevant quantities of proteins useful for cancer treatment have now been expressed specifically in the egg white, with no evidence of ectopic expression or transgene silencing[63].

Two features of retroviral transduction may, however, make it difficult to achieve the high levels of expression desirable for animal pharming applications. The size of the transduced gene is limited to a maximum of approximately 8kb by the capacity of the retroviral particle. This effectively restricts their use to cDNA and small minigene constructs, and severely limits the amount of regulatory and enhancer elements that can be included. Retroviral vectors usually result in single or low copy transgenes, but high trans-

[62] Rapp *et al.* 2003.
[63] Lillico *et al.* 2007.

gene copy numbers are often desirable because they tend to correlate with more abundant expression.

Random integration of proviral DNA also carries the risk that genes adjacent to the integration site may be inappropriately activated, or that integration disrupts an endogenous gene with possible deleterious effects. Retroviruses have, in fact, been successfully used as experimental insertional mutagens. There is also the possibility that, despite extensive precautions taken to disable the retroviral vector, an integrated proviral transgene could somehow recombine with endogenous retroviral elements resulting in a replication-competent virus. While such events are likely to be extremely uncommon, they cannot be absolutely excluded.

In summary, lentiviral transduction offers an efficient means of transgenesis that is likely to become more widely used. Its usefulness in animal pharming will however depend on the levels of expression that can be achieved, a deeper understanding of any possible risks and public acceptance of viral-based vectors.

2.3.2.3 Sperm-mediated gene transfer

This technique could formally be categorized as cell-mediated transgenesis, because a sperm cell is used to introduce exogenous DNA into an oocyte. However it is best regarded as a means of direct nucleic acid transfer into an embryo, because no culture, selection or analysis of cells is carried out before animals are produced. Sperm-mediated gene transfer has been recently reviewed[64].

Since 1971, it has been known that rabbit spermatozoa can associate *in vitro* with exogenous DNA and transfer it to an oocyte by fertilization. Subsequently the same has been shown for sperm cells of other species. In 1989 Marialuisa Lavitrano of the University La Sapienza, Rome, claimed that mouse spermatozoa exposed to exogenous DNA could be used as a vector to generate transgenic mice by artificial insemination. This report stimulated considerable interest because it offered a simple approach to the production of transgenic animals. However, the method has suffered considerable problems of reproducibility and, while there have been reports of transgenic calves and pigs, transgenes introduced in this way frequently undergo rearrangement. This is consistent with findings that DNA which penetrates sperm nuclei becomes fragmented. There have also been reports that DNA introduced directly into the epididymis is transferred to offspring by natural ejaculation and fertilization (reviewed by Sato[65]). The reproducibility and usefulness of this method has yet to be confirmed.

A variation of this technique, based on intracytoplasmic injection of DNA complexed with frozen-thawed or detergent-treated sperm, was devel-

[64] Lavitrano *et al.* 2006.
[65] Sato 2006.

oped in 1999 by Ryuzo Yanagimachi of the University of Hawaii. This has revived interest in the field and several groups have used it to generate transgenic mice, rats and pigs. An outline of the method is given in a recent review[66]. In mice it offers a lower incidence of founder mosaicism and a greater efficiency of transgenesis than standard DNA microinjection, particularly for large transgenes. In pigs, however, the efficiency of transgenesis and number of live born piglets obtained has so far been very low. One problem is failure of the injected sperm head to properly decondense and form a male pronucleus.

In summary, sperm-mediated gene transfer by natural fertilization has not yet proved a reliable method of producing transgenic animals. Variations based on intracytoplasmic sperm injection may offer greater success, but their potential in large animal transgenesis has yet to be fully explored.

2.3.2.4 Embryonic stem cells

Embryonic stem (ES) cells are pluripotent cells derived from early mammalian embryos, first isolated from mice in 1981. ES cells have three defining functional characteristics. Firstly, they can proliferate undifferentiated for extended periods *in vitro*. Secondly, they can differentiate *in vitro*, or as tumours *in vivo*, into cells of the three embryonic germ lineages: ectoderm, mesoderm and endoderm. Thirdly, when reintroduced into a host embryo they are able to participate in development and contribute to all tissues of the animal, including gametes. ES cell research is a large and fast-moving field and the reader is referred to two reviews[67] for further background.

ES cells made possible the engineering of genetic modifications in culture and then study of the effects in whole animals. In the past two decades ES cells have been a powerful tool for the experimental manipulation of the mammalian genome. This work has, however, been largely restricted to mice, and only in mice has an ES genotype been transmitted through the germ line. Efforts have been made to derive ES lines from other mammals and there are reports of ES-like cells in hamster, mink, sheep, cattle, pig, monkey, rabbit, rat and human, but none so far have been germ line competent. Human ES cells remain untestable in this respect for obvious ethical reasons. ES-like cells have also been derived from chickens, but without germ line contribution. Because of their potential advantages, work is ongoing to derive definitive ES cells from livestock species. Recent reports that mouse and human somatic cells can be converted to germ line competent ES cells by the expression of defined genes[68] are particularly interesting, and will aid the extension of ES cell technology to other species.

---

[66] Moreira *et al.* 2007.
[67] Wobus and Boheler 2005; Prelle *et al.* 2002.
[68] Okita *et al.* 2007; Meissner *et al.* 2007; Takahashi *et al.* 2007; Yu *et al.* 2007.

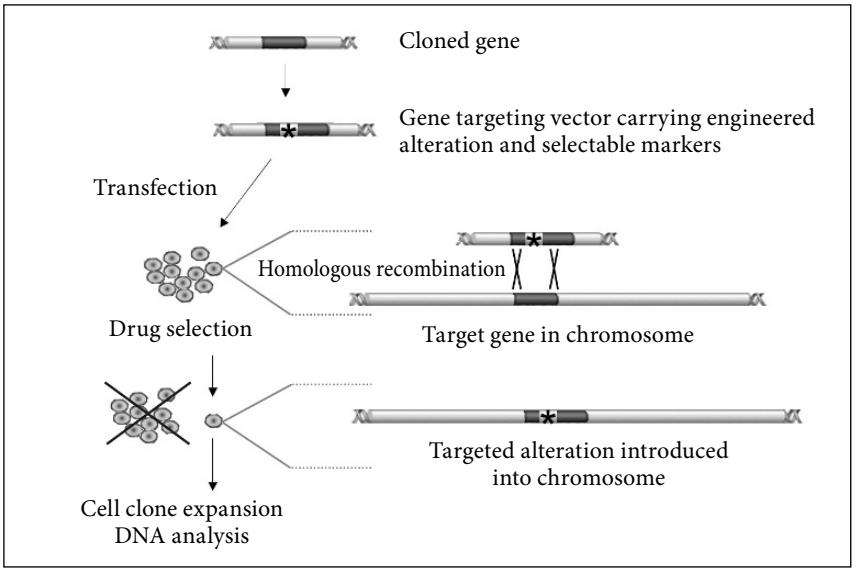


Figure 2.7: Gene targeting by homologous recombination in cultured cells

DNA can be introduced into cultured mammalian cells by a wide range of chemical, electrical, mechanical and viral methods, often collectively referred to as "transfection". In ES cells, transfection can be used to randomly add DNA sequences to the genome, but the most potent application has been the precise modification of genes *in situ*, termed gene targeting, see figure 2.7. Gene targeting exploits the ability of cells to support recombination between exogenous DNA molecules and their cognate chromosomal sequences at regions of shared homology. Typically, ES cells are transfected with a DNA construct carrying an engineered modification flanked by 2–15 kb "arms" of DNA homologous to the target locus. At a certain frequency, transfected cells undergo homologous recombination with the construct and seamlessly incorporate the engineered modification at the target locus. Targeted cell clones can be selected and identified amongst a background of random integrants by a variety of methods.

Isolated targeted ES cell clones can be characterized and then used to generate animals. Gene targeting in mice has been used to inactivate individual endogenous genes by insertion or deletion, replace whole genes, precisely place transgenes in the host genome, introduce subtle gene modifications and to delete megabase-size DNA fragments rendering large regions hemizygous. There are many variations and refinements of the technique, and these continue to produce a wealth of information about many aspects of mammalian biology, see review[69].

[69] Capecchi 2005.

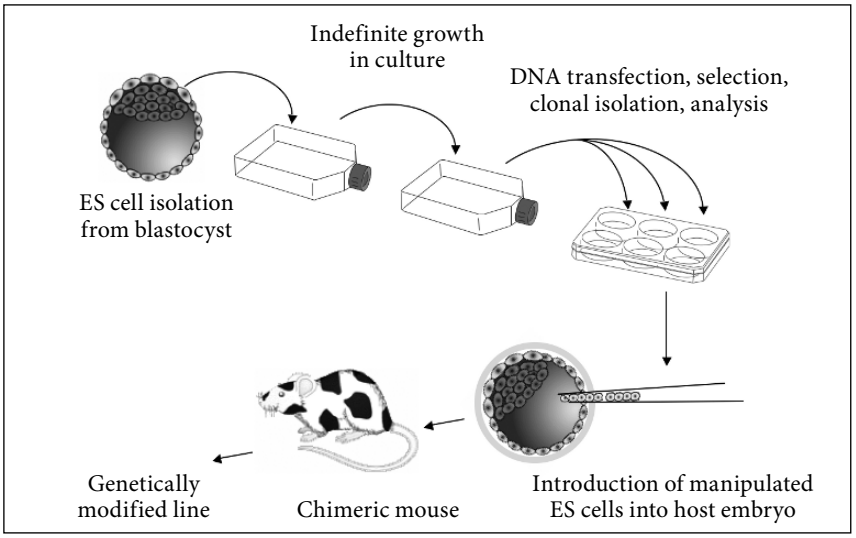


Figure 2.8: Production of genetically modified mice using embryonic stem cells

There are now several possible methods of generating mice from ES cells. The most common exploits the ability of an early embryo to incorporate exogenous ES cells. A small number of ES cells may be aggregated with a pre-morula stage embryo by co-culture in a microwell, or microinjected into the cavity of a blastocyst. Embryos containing ES cells are then transferred to the reproductive tract of recipients to complete development. Animals produced in this way are chimeric, composed of a patchwork of ES and host-derived cells. In mice, chimerism can be readily visualized by marking the ES cells and host embryos with different coat colour genotypes. Chimeras are bred to derive offspring from ES-derived germ cells. See figure 2.8.

Mice derived entirely from ES cells can also be produced by aggregating ES cells with an unviable tetraploid host embryo, generated by prior electrofusion of a two-cell stage embryo. This method is more successful with either early passage, or F1 hybrid ES lines. ES cells have also been used to produce whole animals by nuclear transfer, as described below.

Chicken ES-like cells derived from blastoderm stage embryos have been cultured and transfected in a similar way to mouse ES cells. When injected into the subgerminal cavity of freshly laid eggs, some cells become incorporated into the embryo and contribute to the body. Transfected chicken ES-like cells have been used to generate chimeric hens with a substantial ES contribution, including the egg white producing cells of the oviduct[70], but germ line transmission has not yet been demonstrated.

[70] Zhu *et al.* 2005.

Possible new routes to the production of whole animals using ES cells are opening with reports that gametes can be produced from ES cells *in vitro*. Oocytes and follicular structures have been reported from differentiating mouse ES cells, but it remains unknown whether they can be fertilized[71]. Mouse ES cells have also been shown to form sperm capable of producing viable offspring by ICSI[72]. However, the efficiency of the procedure was low and the offspring had a reduced lifespan, possibly indicating an imprinting problem.

In summary, the importance and usefulness of ES cells are widely acknowledged and considerable research efforts are underway to further understand and manipulate them. This rapidly increasing body of knowledge will likely enable practical ES technology to extend to livestock in the future.

2.3.2.5 Embryonic germ cells

Embryonic germ (EG) cells are undifferentiated cells that resemble ES cells, in that they can be cultured and transfected *in vitro* and then contribute to somatic and germ cells of a chimera. EG cells are derived from isolated primordial germ cells. These are the progenitors of the gametes, and arise outside the embryo and migrate through the body during mid-stage development to their eventual site in the developing gonads, the urogenital ridges. Primordial germ cells cultured with a combination of growth factors: leukemia inhibitory factor, steel factor, and basic fibroblast growth factor, grow and convert to an undifferentiated cell type resembling ES cells.

EG lines were first derived from mice in 1992 and have since been isolated from rat, pig, cattle and chicken. Reintroduction of livestock EG cells into embryos in the same way as ES cells has not been very successful. Porcine chimeras have been produced from both genetically manipulated and normal EG cells. However, the efficiency of chimera formation was poor, EG contribution to each chimera was consistently low and restricted to somatic cells, probably due to imprinting problems[73]. This has restricted their practical usefulness and mammalian EG cells are now rarely used.

Avian EG cells have been more successful, and produced transgenic chickens on several occasions, see review[74]. The typical technique has been to isolate primordial germ cells from the blood of chick foetuses two to three days after laying and convert them to EG cell. Cultured EG cells can be reintroduced into the blood stream of foetuses at the same stage, where they migrate to the gonads and form functional gametes. The efficiency of chimera production and incidence of germ line transmission is increased if

---

71 Hubner *et al.* 2003.
72 Nayernia *et al.* 2006a.
73 Labosky *et al.* 1994.
74 Petitte *et al.* 2004.

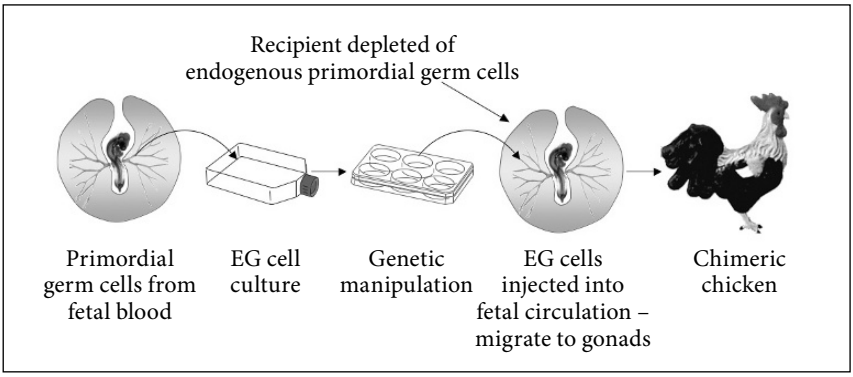


Figure 2.9: Production of genetically modified chickens using embryonic germ cells

recipient eggs are chemically pre-treated to deplete endogenous primordial germ cells (see figure 2.9).

2.3.2.6 Nuclear transfer

The reader is referred to recent reviews[75] for more details of this subject. The replacement of an egg nucleus with that of another cell was first suggested in 1938 by Hans Spemann of the University of Freiburg as a means of determining whether nuclei of differentiated and undifferentiated cells have equivalent development potential. In the 1950s, Robert Briggs and Thomas King in Philadelphia showed that cell nuclei from frog blastocysts could be transplanted to enucleated eggs and direct normal development to feeding-stage larvae, whereas nuclei from mesoderm or endoderm of late-gastrulation stage embryos were unable to do so. This led to the view that development is accompanied by determination of cell fate, a position that was not disproved until three decades later.

During the 1980s, culture conditions and micromanipulation techniques for mammalian embryos improved significantly and nuclear transfer in mammals became a practical proposition. Somewhat unusually, the major developments and breakthroughs were made using livestock rather than mice. Nuclear transfer in livestock is generally carried out by removing the genomic DNA from an unfertilized oocyte, usually by microsurgical withdrawal of a portion of ooplasm containing the second metaphase plate. Enucleation of zygotes has also been used but with less success. The donor nucleus is then introduced into the cytoplast by microinjection or electrofusion. Reconstructed embryos are activated to simulate fertilization, then cultured if possible to identify viable embryos and transferred to the oviduct of foster mothers to complete gestation, see figure 2.10.

[75] Campbell *et al.* 2005; Wells 2005; Vajta and Gjerris 2006.

In 1986, the first viable mammal was produced by nuclear transfer, a sheep produced by fusion of a blastomere of an eight-cell embryo into an enucleated egg[76]. For a decade after, nuclear transfer in mammals was limited to cells obtained directly from early embryos or cultured for very short periods. Nuclear transfer in mice proved to be particularly difficult.

One feature that emerged during this time was the importance of matching the cell-cycle stage of the donor nucleus and the oocyte cytoplasm. Oocytes of most mammalian species pause twice during meiosis, once before the first meiotic metaphase and again at the second, at which stage the oocyte is mature and can be fertilized. Oocyte maturation and arrest are induced by a high level of maturation (or mitosis) promoting factor (MPF). Fertilization causes a chain of events that result in proteolytic cleavage of MPF, breaking the arrested state and allowing the fertilized oocyte to complete meiotic division.

The level of MPF in the oocyte has a profound effect on the outcome of nuclear transfer. If a nucleus is transferred into oocyte cytoplasm with high MPF, the nuclear envelope breaks down and chromatin undergoes chromosome condensation, followed by nuclear reformation and DNA replication. A nucleus from a cell in G1 phase of the cell cycle will undergo normal DNA replication and can support normal development. However, a donor nucleus in S, or G2 phase undergoes aberrant re-replication of DNA, causing aneuploidy or chromosomal damage and consequent failure of development. Nuclear transfer efficiency into unfertilized oocytes can therefore be improved by synchronising donor nuclei in G1. In contrast, if a nucleus is transferred to a fertilized oocyte in which MPF levels have declined, nuclear envelope breakdown does not occur and the cell cycle of an incoming nucleus in either G1, S, or G2 phase will be completed normally. MPF can also be induced to decline by experimentally activating the oocyte by exposure to ionomycin, ethanol, strontium, or by an electrical pulse and these methods have been used to produce live offspring by transfer of S or G2 nuclei.

Cell-mediated transgenesis using nuclear transfer became possible when in 1996, Keith Campbell and Ian Wilmut of the Roslin Institute, Edinburgh reported live sheep produced from embryonic cells cultured for several weeks[77]. They later proposed that the key to successful nuclear transfer was the induction of a quiescent state in the donor cell by serum starvation. Subsequent work revealed that quiescence *per se* is not critical and the effect of serum starvation is to synchronize donor cells in G1 phase. Nuclear transfer was then extended to adult mammary cells. The sheep "Dolly" provided unequivocal evidence that differentiated cells have the capacity to form whole animals, overturning the concept of irreversible determination[78].

---

[76] Willadsen 1986.
[77] Campbell *et al.* 1996.
[78] Wilmut *et al.* 1997.

Nuclear transfer from somatic cells opened a new route to produce transgenic livestock, lifting the requirement for embryonic stem cells for cell-mediated transgenesis. "Ordinary" cells such as primary foetal fibroblasts can be obtained in large quantities, manipulated in culture and then converted into whole animals by nuclear transfer. This could potentially generate large numbers of genetically modified animals without conventional breeding. Initial work in the area was inspired by the possibility of producing such "instant flocks" of animals for pharming. In 1997, Angelika Schnieke of PPL Therapeutics, Edinburgh reported the production of a sheep "Polly" that carried a human clotting factor IX transgene, randomly introduced into the genome by *in vitro* transfection of foetal fibroblasts[79]. Shortly after, this was extended to gene targeting. An α1-antitrypsin transgene was placed into a site chosen as favourable for expression. In both cases transgene expression was directed into milk[80].

Somatic cell nuclear transfer has now been used to generate transgenic and gene targeted animals in several other species, demonstrating that sophisticated genetic manipulations are possible in livestock. However, so far relatively few gene-targeted animals have been generated. Genes that have so far been targeted in livestock are: α1 type1 collagen, α1-3 galactosyltransferase, immunoglobulin-μ, and PrP and CFTR. The main difficulty is that primary somatic cells are short-lived in culture, allowing little time for cell transfection, selection and clonal expansion. This obstacle can in part be overcome by "rejuvenating" cells by nuclear transfer and rederivation from resulting foetuses. Such a procedure also allows successive rounds of *in vitro* genetic manipulation to be carried out relatively quickly, but does introduce the risk that genetic aberrations occur in the cultured cells, but remain undetected until animals are born.

Foetal and perinatal mortality and morbidity is the most serious issue that faces nuclear transfer technology. The severity of the problem varies between species, cell types and experimental regimes and is unrelated to genetic manipulation of the cultured cells. Rather, it is believed to be a consequence of defective epigenetic reprogramming of the donor nucleus and possibly incompatibility between the cell-derived nuclear genome and the oocyte-derived mitochondria. Cumulative data for cattle collected up to 2005 indicate that more than 1,500 cloned calves were born, of these 60–70 % survived normally to adulthood. The performance of these, including reproduction, was similar to non-cloned animals. Evidence is also accumulating that ill effects are limited to the first generation. Offspring, including those from two nuclear transfer parents, exhibit no increased morbidity or mortality.

---

[79] Schnieke *et al.* 1997.
[80] McCreath *et al.* 2000.

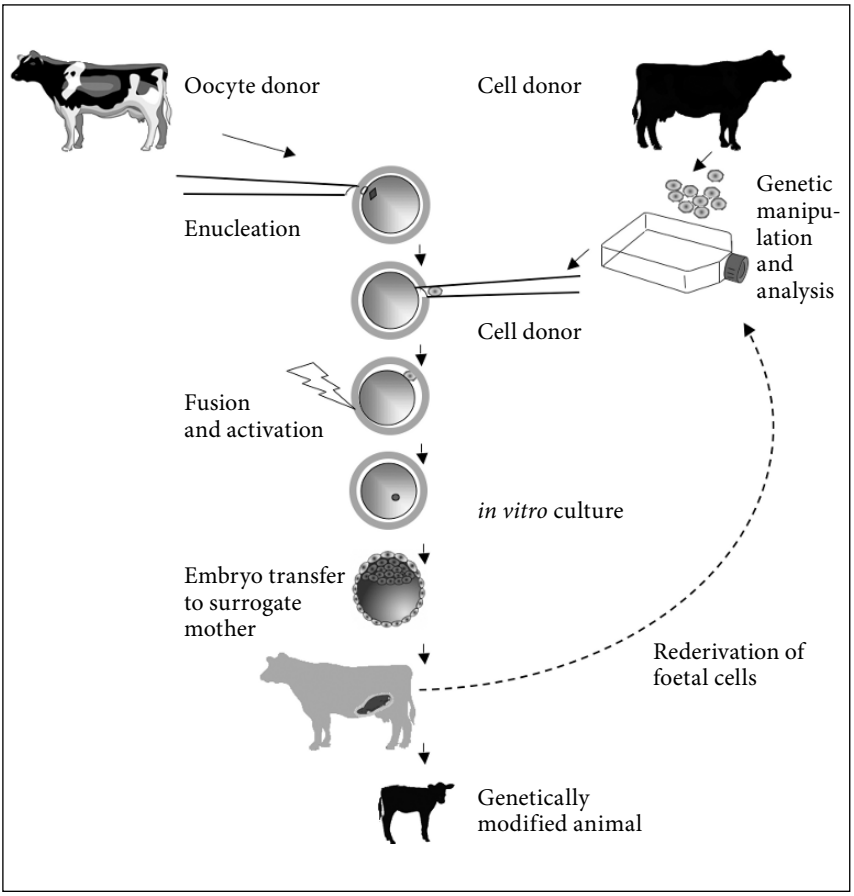


Figure 2.10: Generation of transgenic animals by nuclear transfer

Extension of nuclear transfer to mice[81] was an important development, because it allowed ES cells to be used as nuclear donors. ES cells were found to be significantly superior to somatic cells, both in the efficiency with which animals are derived and also the viability and health of cloned animals. One explanation is that the pattern of gene expression in an ES cell nucleus resembles an embryonic blastomere more closely than does that of a highly differentiated cell such as a fibroblast, and consequently requires less extensive reprogramming. However, recent analysis has questioned whether the differentiated state of the donor cell correlates with success of nuclear transfer[82].

In summary, nuclear transfer from somatic cells has yet to fulfil its initial promise of producing instant flocks or herds, although efficiencies are

[81] Wakayama *et al.* 1998.
[82] Oback and Wells 2006.

continually improving. At present, nuclear transfer offers the only practical method of gene targeting in livestock. Clone viability remains a problem, but technical refinements and the prospect of livestock embryonic stem cells as nuclear donors may yet resolve this.

2.3.2.7 Spermatogonial stem cells

Spermatogonial stem cells (SSCs) are a self-renewing population of germ cells within the adult testes that form the spermatogonia and differentiate to spermatozoa. In the early 1990s, Ralph Brinster of the University of Pennsylvania showed that SSCs from one mouse could be transferred to the testes of another sterile or sub-fertile mouse, where they locate to a stem cell niche in the seminiferous tubules and go on to produce functional sperm. Subsequently Brinster and others showed that SSCs could be transferred across species. Rat SSCs could be transplanted into mouse testes and produce sperm and *vice versa;* hamster SSCs also produce sperm in mouse testes. More distantly related species, such as rabbit, pig, baboon, and human, also populate mouse testes, but do not complete spermatogenesis. It is possible that this barrier could be overcome by co-transplantation of sertoli cells, or testis tissue. Transfer of germ cells between livestock species is now being developed. Ina Dobrinksi of the University of Pennsylvania reported in 2002 the transfer of germ cells between pigs, although sperm were not produced, and in 2003 the transfer of germ cells marked with a transgene between goats and transmission of the transgene through sperm to offspring, see review[83].

Germ cell transplantation offers an exciting new method of producing genetically modified animals, providing culture conditions are developed under which SSCs can be expanded, undergo DNA transfection and selection, while remaining genetically and epigenetically intact for transplantation. Because modifications are introduced directly into the male germ line, without embryo manipulation, the time required for gestation and maturing founder animals is avoided, a significant factor in large animals. In early work, Brinster demonstrated that SSCs could be cultured *in vitro* for several months while retaining the ability to repopulate a recipient testis, but they proliferated poorly due to inhibitory factors in serum. This problem severely limited their usefulness for several years. In 2004, glial cell line-derived neurotrophic factor (GDNF) was identified as promoting SSC self renewal *in vivo.* Subsequently, serum-free conditions including GDNF that support SSC self-renewal *in vitro* were developed. In 2006, a group led by Takahashi Shinohara of the University of Kyoto reported targeted inactivation of a gene in cultured mouse SSCs, their transplant into testes and transmission of the targeted allele through sperm to offspring[84]. Researchers differ in their findings regarding the most suitable SSC culture media.

---

[83] Dobrinski 2005.
[84] Kanatsu-Shinohara *et al.* 2006.

Interestingly, SSCs themselves offer a source of primitive pluripotent cells, apparently functionally equivalent to ES cells. In 2004, Shinohara used specific culture conditions to establish cells from neonatal mouse testis that were phenotypically similar to ES cells[85]. These differentiated into various types of somatic cells *in vitro* and, most importantly, formed germ line chimeras when injected into blastocysts. Other researchers have made similar findings, reporting that a subset of cultured SSCs converted to an ES-like morphology in the presence of particular growth factors[86]. Again, these differentiated *in vitro* and early stage derivatives could form germline chimeras.

In summary, this is a promising new field. SSC technology is, however, at an early stage and the techniques required for transgenesis, such as conditions for extended culture, are still being developed.

2.3.2.8 Adult stem cells

The nature and differentiative abilities of adult stem cells, for example haematopoietic, amniotic and mesenchymal stem cells, are the subject of active research with frequently contentious results. Most of the effort in this field is directed towards generating human tissue for regenerative medicine. However, the development of methods that support *in vitro* culture and transfection of adult stem cells may also facilitate their future use in cell-mediated transgenesis. This could be in combination with nuclear transfer as has already been shown for mesenchymal stem cells[87], or as a source of gametes, if recent reports that male germ cells can be derived from bone marrow stem cells[88] are confirmed.

2.3.2.9 Overview

Figure 2.11 (p. 49) outlines the stages and procedures involved in the establishment of a transgenic production herd. Table 2.5 summarizes the various methods of transgenesis.

2.3.3 Choice of species and site of production

In mammals, research and development of animal pharming projects has overwhelmingly been carried out in mice. Species used for production are: rabbit, sheep, goat, pig and cattle. The choice of production species is made on the basis of a combination of factors, such as ease of husbandry, generational interval, fecundity and the potential yield of recombinant protein. Reproduction data and production capacity of different mammalian species in milk are shown in table 2.6 below (p. 50–51).

---

[85] Kanatsu-Shinohara *et al.* 2004.
[86] Guan *et al.* 2006.
[87] Kato *et al.* 2004; Bosch *et al.* 2006.
[88] Nayernia *et al.* 2006.

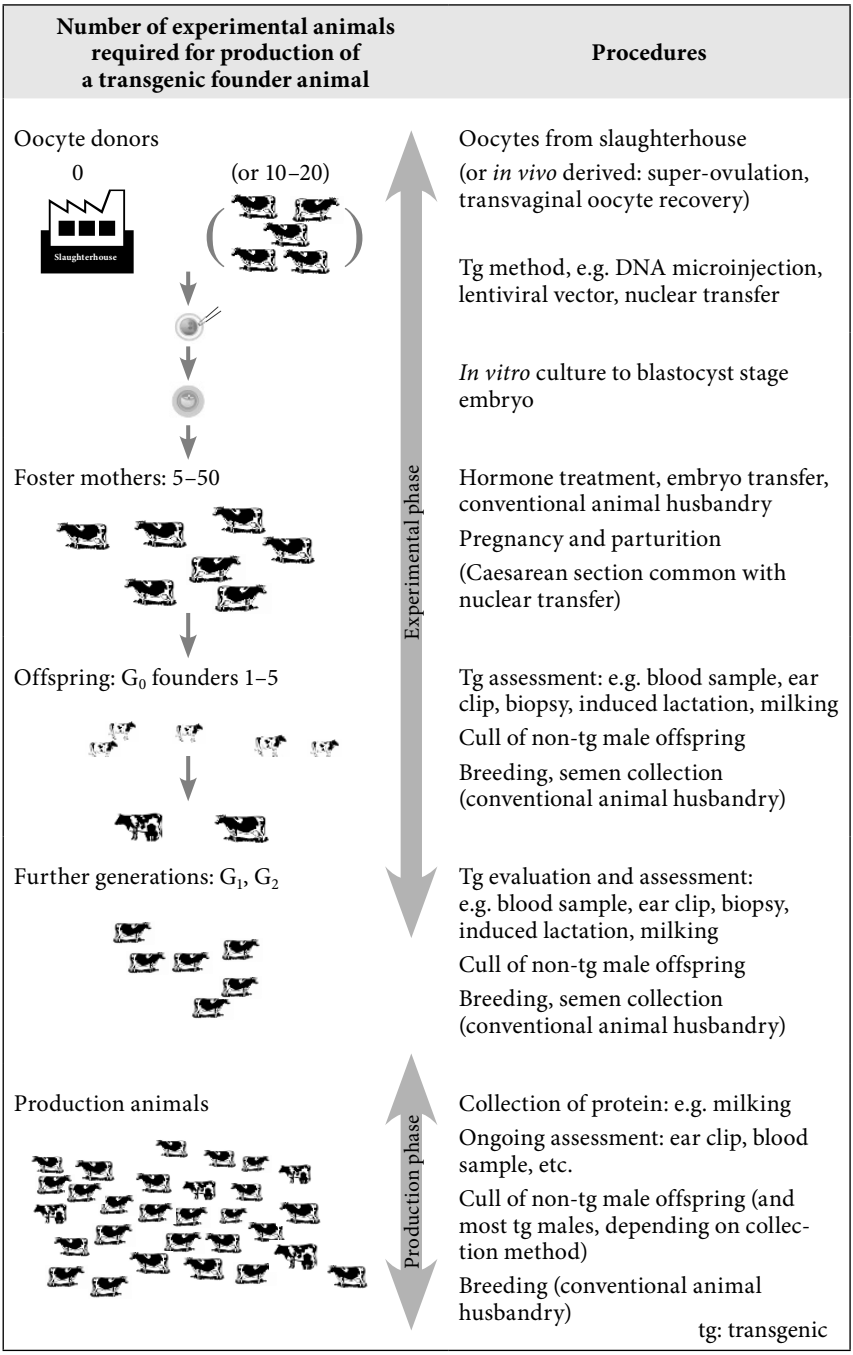


Figure 2.11: Establishing a transgenic production herd: animals and procedures required

Table 2.5: Methods of producing transgenic animals

Method	Transfer route	Additional DNA	Gene targeting	Founder anima I	Germline transmission	Inadvertent health effects	Demonstrated in mammalian species species	Efficiency in livestock (oocyte/live offspring[4])
DNA Microinjection	DNA => zygote	No	No	Mosaic possible, multiple integrations rare	Yes, reduced by mosaicism	Very rare, insertional mutagenesis possible	Mouse, cattle, pig, sheep, goat, rabbit	0.5–1 %
Retroviral Transduction	Viral vector => packaging cells => infectious virus => zygote or oocyte	Retroviral sequences	No	Mosaic possible, multiple integrations common	Yes, reduced by mosaicism	Very rare, insertional mutagenesis or gene activation possible	Mouse, cattle, pig	50–80 %
Sperm-Mediated Transfer	DNA => sperm => natural fertilisation or ICSI	No	No	Fragmented transgenes common by fertilisation	??	Insufficient data	Mouse, pig	34 %[3]
Embryonic Stem Cells (ES)	DNA=> ES cells => incorporate into diploid embryo	Selectable marker	Yes	Chimera	Yes, reduced by chimerism	Very rare, insertional mutagenesis possible	Mouse,	Not done
Embryonic Stem Cells (ES)	DNA=> ES cells => incorporate into tetraploid embryo	Selectable marker	Yes	Completely ES-derived	Yes	Very rare, insertional mutagenesis possible	Mouse	Not done

Continued on next page

Table 2.5: Methods of producing transgenic animals

Method	Transfer route	Additional DNA	Gene targeting	Founder animal	Germline transmission	Inadvertent health effects	Demonstrated in mammalian species	Efficiency in livestock (oocyte/live offspring[4])
Embryonic Stem Cells (ES)	DNA → ES cells → form male or female gametes → fertilisation	Selectable marker	Yes	Heterozygous for ES genotype	Yes	Imprinting problems, poor development, low viability	Mouse	Not done
Nuclear Transfer	DNA → cells → nuclear transfer	Selectable marker	Yes	Nuclear genome completely cell-derived, mitochondria from oocyte	Yes	High founder mortality, morbidity. Subsequent generations OK	Mouse, sheep, pig, cattle, goat[1]	0.5–5%
Spermatogonial Stem Cells (SS)	DNA → SS cells → incorporate into embryo	Selectable marker	Yes	Chimera	Unknown	Unknown	Mouse	Not done
Spermatogonial Stem Cells (SS)	DNA → SS cells → transplant into testis → fertilisation	Selectable marker	Yes	Heterozygous for SS genotype	Yes	Insufficient data	Mouse, goat	?%[3]
Embryonic Germ Cells (EG)	DNA → EG cells → incorporate into embryo	Selectable marker	Yes	Chimera	Not demonstrated in mammals[2]	Imprinting problems	Mouse	Not done

Notes: 1. Use of F1 hybrid ES cells as nuclear donors shows significantly reduced mortality and morbidity in mice. 2. Germline transmission of EG genotype demonstrated in chickens. 3. Data from one laboratory. 4. In large animals, oocytes and zygotes are now almost always derived from slaughterhouse material. The efficiency of the procedure, therefore, primarily affects the number of animals required as recipients to gestate manipulated embryos.

Table 2.6: Basic reproductive data for livestock mammals

Species	Gestation period (months)	Age at sexual maturity (months)	Number of offspring	Age at first lactation (months)	Recombinant protein production (kg/individual/yr)
Cattle	9	16	1	33	40–80
Goat	5	8	1–2	18	4
Pig	4	6	~10	16	1.5
Sheep	5	8	1–3	18	2.5
Rabbit	1	5	~8	7	0.02

In birds, both research and production have focused on the domestic chicken. A modern hen produces more than 300 eggs per year, and the relatively short time to sexual maturity (circa 5 months) allows for rapid expansion of transgenic flocks.

As outlined in the introduction, the main reason to choose to express a particular protein in animals or cultured animal cells, rather than bacteria, plants or yeast, is because functional and/or immunological properties require addition of appropriate sugar chains (oligosaccharides) to the amino acid chain. This process is termed glycosylation.

The pattern and type of protein glycosylation vary widely between microorganisms, plants and mammals. Oligosaccharide groups exist as either short chains linked to either serine or threonine amino acid residues in the protein chain (O-linked glycosylation), or as longer complex branching chains linked to asparagine (N-linked glycosylation). Many mammalian proteins require N-linked glycosylation for correct folding and stability; most bacteria are unable to do this. Plants can carry out N-linked glycosylation, but the sugars added are frequently very different to those present on mammalian proteins. Notably, plants do not add a group of sugars termed sialic acids, which frequently terminate oligosaccharide chains on glycoproteins. Glycosylation patterns also vary between mammalian species, tissue types and even between metabolic states of the same tissue. Importantly, humans differ from the majority of other mammals in the type of sialic acids present. The two principle forms are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Humans have a mutation in the enzyme responsible for producing Neu5Gc and therefore lack this form. Oligosaccharide analysis of immunoglobulins from different species illustrates these differences.

Only Neu5Ac: Human, chicken
Mainly Neu5Ac: Guinea pig
Mainly Neu5Gc: Rat, rabbit, dog, cat
Only Neu5Gc: Cattle, sheep, goat, horse, mouse, rhesus monkey

Expressing a protein in a species or tissue different from its normal location may therefore result in altered glycosylation. The importance and possible pharmacological consequences of differences in glycosylation must be thoroughly investigated by molecular, biochemical and physiological analysis, and ultimately by clinical trial. Two important considerations are the effects on pharmokinetic properties, i.e. activity, rate of clearance from the body and immunogenicity. It is known that humans carry antibodies that recognize Neu5Gc.

Glycosylation, while important, is however only one of many possible modifications that may be required for correct protein function. These include: propeptide cleavage, multichain assembly, disulphide bonding, phosphorylation, hydroxylation, amidation, methylation, hydroxylation, γ-carboxylation, acylation and lipid attachment. The repertoire of enzymes that carry out these functions varies considerably between mammalian tissue types. Ideally, the protein processing capability of the producing cells should match the requirements of the desired protein, or be readily modifiable to carry out the appropriate processing. The current state of the art, however, offers only a limited choice regarding site of production in transgenic animals, and the ability of different tissues to express and process exogenous proteins has not been comprehensively studied.

In birds, animal pharming has focused exclusively on protein production in the white of eggs. In mammals, four sources of exogenous proteins have been studied to date, each of which are body fluids. These are: milk, urine, seminal fluid and blood. Fluids are more suitable than solid tissues for this purpose because they are renewable and can be obtained without harm or excessive invasion. Furthermore, many biomedically important proteins are themselves secreted into body fluids. Milk is by far the best studied of these production systems and the only method that has been examined on a large-scale.

2.3.3.1 Milk

Milk was a natural focus for the development of animal pharming, see review[89]. The lactating mammary gland has a huge capacity to synthesize proteins and other biochemicals for infant nutrition. The dairy industry is well established and scientifically advanced, not only in cattle but also sheep and goats. The necessary equipment and expertise required for the collection, processing and early stage purification of transgenic milk are therefore readily available.

The major milk proteins are caseins, with five types in mice, four in sheep and cows and two in human. Caseins are hydrophobic and associate into spherical complexes that form a fatty suspension. The soluble, or whey, fraction of milk contains hydrophilic proteins that differ between mam-

[89] Clark 1998.

malian species. Whey acidic protein (WAP) is the major whey protein in rodent milk and is also present in pigs, while β-lactoglobulin (BLG) is the major whey protein in sheep, goats and cows; both are absent from human milk. α-lactalbumin has a role in lactose synthesis and is present in the whey of all milks that contain lactose.

In 1989 John Clark of the Roslin Institute, Edinburgh, demonstrated that the BLG promoter could be used to direct the expression of the human blood clotting Factor IX gene in sheep and that the product was secreted during lactation[90]. Since then, the promoters and regulatory sequences of almost all major milk genes have been utilized and investigated for their suitability in driving the expression of potentially useful proteins.

A large number and wide variety of foreign proteins have been expressed in the milk of transgenic animals and expression levels as high as 35g/L have been achieved[91]. In each case the foreign protein is secreted as part of the whey fraction. This work has included: complex multichain proteins, for example fibrinogen; combinations of transgenes designed to supplement the natural protein processing abilities of the lactating mammary gland, for example prolyl hydroxlyase co-expressed with type 1 procollagen; and co-expression of transgenes to improve the stability of secreted protein in milk, for example α1-antitrypsin protease inhibitor with fibrinogen.

Hundreds of transgenes have been "trialled" in the lactating mammary gland, with the great majority in mice. Pilot studies in mice provide an indication of the feasibility of a large animal study and indicate whether any adverse effects to animal health can be expected, an important issue in the expression of highly bio-active recombinant proteins such as erythropoietin. It is difficult to provide a definitive list of proteins expressed in milk, because some of this work has been carried out by companies and not made public. Those published, or otherwise known to the authors, are listed below:

- Anti-microbial proteins: Lysozyme, lactoferrin, tissue non-specific alkaline phosphatase, lysostaphin, antimicrobial peptides for example β-defensins.
- Blood clotting and anti-clotting factors: Antithrombin III, protein C, factor VII, factor VIII, factor IX, fibrinogen, tissue plasminogen activator, hementin, urokinase, thrombin activated plasminogen.
- Cell surface proteins expressed in soluble form: CD4 (HIV receptor), transferrin receptor, cystic fibrosis transmembrane conductance regulator, intercellular adhesion molecule 1 (human rhinovirus receptor), pentraxins for example serum amyloid P and C-reactive protein.
- Cytokines and growth factors: Erythropoietin, Interleukin 2, Interleukin 10, thrombopoietin, insulin-like growth factor 1, nerve growth factor b, granulocyte colony stimulating factor, Interferonγ.

---

90 Clark *et al.* 1989.
91 Wright *et al.* 1991.

- Detoxifying enzymes: Butyrylcholinesterase.
- Digestive enzymes: Bile salt stimulated lipase.
- Hormones: Follicle stimulating hormone, lutenising hormone, parathyroid hormone, growth hormone, leptin.
- Milk proteins: α-lactalbumin, αs1-casein, β-casein, κ-casein, β-lactoglobulin, whey acid protein.
- Immunoglobulins: Many types of single chain antibodies and also monoclonal antibodies composed of both light and heavy chains.
- Protease inhibitors: α1-antitrypsin, lung elastase inhibitor, C1 inhibitor (complement system inhibitor).
- Protein modification enzymes: These are usually co-expressed with other products. Furin, prolyl hydroxylase, glycosyltransferases.
- Peptides: These are usually expressed as a fusion with another carrier protein. Calcitonin, amylin, anti-microbial peptides.
- Structural proteins: Type 1 collagen, type 2 collagen, spider dragline silk.
- Viral and microbial proteins for vaccine production: Rotavirus Vp2 and Vp6 antigens, malaria parasite surface antigens, hepatitis B virus surface antigen.
- Others: Transferrin, serum albumin, alpha-fetoprotein, hepatocarcinoma-intestine-pancreas/pancreatic-associated protein, n-3 fatty acid desaturase, stat5 transcription factor, endostatin, zona pellucida glycoprotein 3, acid alpha-glucosidase, extracellular superoxide dismutase, pulmonary surfactants B and C, n-3 fatty acid desaturase.

Of the subset of these proteins investigated in livestock, only a very small number have continued to preclinical and clinical trials and only one product has so far gained regulatory approval. Those currently in commercial development are summarized in table 2.7.

A significant drawback to milk is that it is a complex and rich mixture of proteins, lipids and carbohydrates. Therefore, purification of the desired protein requires multiple steps which can be costly. Protein purity is of paramount importance where the protein product is to be administered to patients on a long-term basis, especially intravenously, because even minute quantities of contaminating milk components could be immunogenic.

Purification can be circumvented in those special applications where milk is to be ingested as a nutraceutical. For example, transgenic goats have been produced that secrete enhanced amounts of the anti-microbial protein lysozyme into their milk. The intention is to mimic human breast milk, which is also rich in lysozyme, to alter gut flora and combat gastrointestinal microbial infections[92].

[92] Maga *et al.* 2006.

Table 2.7: Proteins produced in milk currently in commercial development[93]

Product	Animal	Company	Indication/ application	Development stage
Anti-thrombin III (ATryn®)	Goat	GTC, US	Antithrombin deficiency	Approved in EU. Phase 3 clinical trials in US
C1 inhibitor	Rabbit	Pharming, Holland	Hereditary angiodema	Phase 3 clinical trials
Alpha fetoprotein	Goat	Merrimack and GTC, US	Rheumatoid arthritis	Phase 2 clinical trials
Alpha glucosidase	Rabbit	Pharming, Holland	Pompe's disease	Phase 2 clinical trials, on hold
Growth hormone	Cow	Biosidus, Argentina	Dwarfism	Preclinical
Lactoferrin	Rabbit	Pharming, Holland	Infection inflammation	Preclinical
Collagen	Rabbit, Cow	Pharming, Holland	Various biomaterials	Preclinical
Fibrinogen	Rabbit, Cow	Pharming, Holland, GTC, US	Tissue sealant	Preclinical
Albumin	Cow	GTC, US	Excipient, blood expander	Preclinical
Alpha 1 antitrypsin	Goat	GTC, US	Hereditary AAT deficiency	Preclinical
Malaria vaccine	Goat	GTC, US	Malaria	Preclinical
CD137 antibody	Goat	GTC, US	Solid tumours	Preclinical
Rotavirus pseudoviral particles	Rabbit	BioProtein, France	Antigen carriers for vaccine	Preclinical
Butyrylcho-linesterase	Goat	Pharmathene, US	Organophos-phate poisoning	Preclinical

2.3.3.2 Urine

The mammary gland has proved to be an unsuitable site for the production of some highly bioactive proteins, such as growth factors or cytokines, because they can enter the general circulation and affect the physiology of

[93] Data from Biopharm International 1st August 2006 and Nature Biotechnology (2006) 24:877.

the animal. In contrast, the contents of the bladder, being potentially noxious, are sequestered from the body.

A system to express foreign proteins in urine was developed in the late 1990s using the uroplakin genes[94]. Uroplakins are membrane-associated proteins expressed specifically in the differentiated uroepithelium of the bladder and urethra. The mouse uroplakin II gene promoter has been used to direct expression of human growth hormone (hGH) and also human granulocyte macrophage-colony stimulating factor (hG-CSF) in mice. Production in the kidney has also been investigated using the gene promoter of Tamm Horsfall protein, also called uromodulin, which is expressed and secreted from the epithelium of the ascending loop of Henle. Mice expressing and secreting hGH into urine have been produced.

It is not yet clear how appropriate urine is as a source of bioactive proteins. Although the body may not be exposed to the natural contents of the bladder, segregation of transgenic proteins secreted into this compartment still depends on the tissue specificity of the gene promoters. Ectopic transgene expression may lead to circulating proteins. Notably, both the uroplakin II hG-CSF mice and the Tamm Horsfall hGH mice showed evidence of transgene protein in peripheral blood.

The greatest problem with this method of production is, however, the low synthetic capacity of bladder and kidney, which is far less than the mammary gland. The yield of protein per ml is therefore very low, in the order of ng/ml. While this may be suitable for certain high value proteins, the practical usefulness of the system remains to be demonstrated. Proponents do, however, point out that low yield is partially compensated by the large volume of urine obtained from animals such as cattle. Unlike milk, urine is produced during the lifetime of the animal independent of age, sex and lactation. Furthermore, because urine contains little protein and lipid, product purification should, in theory, be simpler than from milk. The stability of recombinant protein in urine is, however, a potential problem that remains to be fully explored.

Work published so far has been restricted to mice, although there have been preliminary reports of transgenic pigs expressing hGH in urine.

2.3.3.3 Seminal fluid

Porcine seminal fluid has been suggested as a suitable source for bioactive proteins[95]. Proponents state that the accessory male sex glands have a substantial protein synthetic capacity, semen is available in reasonably large volumes (200–300 ml per ejaculate) and, because protein secretion is strictly exocrine, bioactive proteins could be produced without adversely affecting the animal. This work is at an early stage and determination of its useful-

[94] Kerr *et al.* 1998.
[95] Dyck *et al.* 1999.

ness will require further investigation. This will include identifying appropriate genes and sequences to drive secretion into seminal fluid. Possible candidates are the spermadhesins, the major protein component in porcine semen. Other important factors are the protein processing capacity of the producing tissue, stability of foreign proteins in semen and ease of product purification.

2.3.3.4 Blood

The physiology and development of the animal are highly exposed to any adverse effects of bioactive proteins circulating in the blood, therefore the range of suitable products is very restricted. Human haemoglobin for use in synthetic blood substitutes has been produced in pigs by the company DNX of Princeton, New Jersey[96], but this was discontinued because of difficulties purifying the human protein away from the very similar porcine protein.

Production in the blood of transgenic livestock will likely gain prominence as a source of human polyclonal antibodies. Progress is being made towards the production of animals with humanized immune systems[97]. Such animals could, in principle, be immunized against a wide range of antigens to provide an abundant source of human polyclonal antibodies. These are likely to play an important role in passive immunotherapy in the future and offer considerable advantages over monoclonal antibodies. For example, they are more effective than monoclonals in immune complex formation and better mimic the natural immune response; they can also disable pathogens which require neutralization of multiple epitopes, pathogens with diverse strains and venoms with multiple toxic components. Importantly, polyclonal antibodies can only be produced in people or transgenic animals. Most applications would require large animals for production of adequate quantities of serum.

2.3.3.5 Bird eggs

Chicken eggs have several advantages that make them attractive for the production of foreign proteins. The poultry industry is well developed and modern breeds of chickens are highly productive, laying about one egg per day. Collection of eggs is very simple and can be scaled up easily. Production is also very flexible, large flocks of birds can be rapidly produced from a single transgenic male. Furthermore, the use of eggs for pharmaceutical purposes is already established for the production of vaccines, providing a framework of regulatory guidelines for good manufacturing practice.

Production of therapeutic proteins in eggs is less advanced than production in milk, because of the technical problems of avian transgenesis. No products are as yet in the commercial pipeline, but several companies

---

[96] Swanson *et al.* 1992.
[97] Kuroiwa *et al.* 2002; Jakobovits *et al.* 2007.

are actively pursuing product development. Proponents point out that the chicken may be more suitable than mammalian systems for certain proteins. For example, some bioactive proteins with toxic effects in mammals may not affect birds. There is also evidence that chickens and human proteins have similar glycosylation patterns, as discussed in earlier, however current data are restricted to a few proteins and considerably more information will be required to assess the system properly.

The secretory cells of the chicken oviduct certainly have a high protein synthetic capacity. Each egg contains approximately 4g protein in the white, of which more than 54% is ovalbumin. Other major protein constituents are ovotransferrin (12%), ovomucoid (12%) and lysozyme (3.4%). This low protein complexity should simplify purification, while natural protease inhibitors present in albumin may also help stabilize foreign proteins.

Chicken ES-like cells transfected with an ovalbumin gene construct, containing 7.5–15kb of the ovalbumin 5' regulatory sequences that direct expression of human immunoglobulin heavy and light chains, have been used to generate somatic chimeric hens that secreted biologically active antibody into the egg white[98]. However, there was evidence of ectopic transgene expression. This was not observed in more recent experiments[99] which used lentiviral vectors containing only about 3kb of regulatory sequences for the expression of an interferon or a mini-antibody for cancer treatment.

2.3.4 Production of proteins from transgenic animals

2.3.4.1 Analysis of transgenic animals

Analysis of integrated transgenes. In 1995, the United States Food and Drug Administration (FDA) produced guidelines for pre-market data submission for potential products from transgenic sources. Amongst other specifications, these require that the structure and expression pattern of the integrated transgene construct be characterized in the founder animal and demonstrated as reliable through subsequent generations.

Each animal line destined for commercial production should be analysed to determine the structure, integrity, copy number and integration site of each integrated transgene. This analysis will include Southern hybridization of the genomic DNA to identify the lengths of various restriction fragments predicted from the construct structure. Fluorescent *in situ* hybridization of metaphase chromosome spreads can also be employed to identify the chromosomal location(s) of integrated transgenes. Molecular cloning of the integrated transgene and its proximal flanking regions may be required to determine the DNA sequence of the integrated transgene locus.

---

[98] Zhu *et al.* 2005.
[99] Lillico *et al.* 2007.

DNA introduced into mammalian embryos by microinjection tends to integrate as tandem repeats generally oriented head to tail and usually, but not always, at a single locus randomly located in the host genome. Introduction of transgenes by cell transfection has broadly similar results, but often leads to a more complex transgene array at the integration site. Lentiviral vectors also integrate randomly but as single copies at each integration site. Multiple copies of a viral transgene in a founder animal will therefore segregate in subsequent generations in Mendelian fashion.

Transgene loci produced by random cell transfection differ from those produced by DNA microinjection because selectable marker genes are necessarily introduced with the transgene; these will typically encode resistance to a commonly used antibiotic, for example G418, blasticidin or puromycin. To avoid possible gene flow from the transgenic animal to prokaryotes, bacterial gene promoters are excluded from the selectable marker genes. Antibiotic resistance genes can also be flanked by site-specific recombination elements, such as *loxP* substrate sites for *Cre* recombinase, allowing their removal. However, in multiple arrays this may result in large deletions.

Transgene loci produced by gene targeting are quite distinct from random events, in that a correctly targeted locus will carry a single copy of the predetermined engineered change and the rest of the genome is left unaltered. An antibiotic or other selectable marker gene is necessarily included at the target site, but again can be removed by site-specific recombination if necessary.

Multiple transgenes that are co-injected or co-transfected generally co-integrate at the same locus. Founder animals carrying high transgene copy numbers are frequently chosen to establish transgenic lines because they often produce the most abundant levels of expression, but it has been observed that such lines can undergo transgene silencing or recombination and copy loss over generations. Transgene copy loss occurs most frequently where elements in a tandem array are in inverse relative orientation. Such configurations tend to be unstable and can lead to deletions, duplications and incomplete genes. Incomplete genes are particularly undesirable because, where breaks occur within coding sequences, shifts in the translational reading frame can lead to the expression of truncated and/or aberrant protein species. Arrays of multiple transgenes can be complex to analyse.

Analysis of transgene mRNA expression. The pattern of transgene expression should be characterized to determine its tissue specificity. This is primarily for the benefit of the producing animals, to assess whether any undue deleterious effects are likely to arise as a consequence of inappropriate transgene expression. Samples of a wide variety of tissue types obtained by necropsy of transgenic animals are analysed by reverse transcriptase PCR (RT-PCR),

or Northern hybridization to detect spatial, or temporal ectopic expression of the transgene. The significance of any ectopic transgene expression will depend on the level and site of expression and the nature of the encoded protein.

Transgene mRNA expressed by the appropriate tissue should be rigorously characterized to identify the full range of mRNA species present. This is necessary to determine the integrity of the mRNA and whether it is correctly spliced. Aberrant mRNAs, even if present as only minority species, can encode aberrant proteins with possibly significant clinical consequences.

Analysis of transgene protein expression. The aims of transgenic protein analysis are to determine: whether a protein is fully functional, if degradation occurs for example in milk, and whether expression levels are sufficient for commercially viability. One then has to investigate to what extent the transgenic recombinant protein product resembles the native form, and whether any differences affect function, stability, half-life and immunogenicity. To this end considerable efforts will be made analysing protein products by functional assay, mass spectroscopy, peptide mapping, protein sequencing and glycoprotein analysis.

Clearly, any human pharmaceutical product should be of consistent quality. Variations in expression level can affect protein structure. For example, if the post-translational modification capacity of the producing tissue is limited, high levels of expression may exceed that limit and result in partially or unmodified protein and altered bioactivity. The amount of protein produced by individuals in a transgenic herd or flock should, therefore, vary as little as possible. Acceptable upper and lower limits should be set to allow standardization and quality assurance of the purification process.

The purity of the protein preparation will clearly be an important factor in the assessment of any transgenic product. This is especially important where it is to be administered intravenously. Producers must ensure removal of host animal proteins and DNA, chemical reagents and ensure exclusion of potential pathogens such as microorganisms, viruses and prions.

Collection, processing and protein purification. Basic collection and processing methods for large quantities of milk and eggs are well established. Collection procedures suitable for bulk collection of other fluids such as urine or semen have yet to be devised.

Large-scale recombinant protein purification has so far only been developed for milk. This is a multi-step process that combines standard methods developed for the dairy industry with procedures developed for purification of recombinant proteins produced in cell culture. The level of purity required of a particular product is determined by its application. If

the protein is to be ingested as a nutraceutical, then skimmed milk could be suitable. If, however, the product is to be injected intravenously on a regular basis over long periods, then very high levels of purity would be required.

The nature of the protein will determine the specifics of its purification. As most recombinant proteins are present in the whey fraction, the first steps are removal of fat and suspended caseins by procedures that may include: centrifugation, acid or PEG precipitation or chymosin treatment and/or microfiltration. This would then be followed by a series of chromatography steps to isolate the recombinant protein away from the whey, remaining milk proteins and other contaminants. Final clean-up steps might include ultrafiltration and possibly heat treatment to prepare a pharmaceutical-grade therapeutic product. Current experience indicates a final yield of purified product of between 40–60 % of the amount in milk, depending on the nature of the protein and the required purification procedure. The greatest loss tends to be during casein removal. This may be reduced by treatment with chelating agents that deform casein micelles and release associated recombinant protein.

Standards for processing plants are equivalent to those already established for the purification of recombinant proteins from cell culture, or native proteins derived from human sources such as blood. Requirements for the process included validation for product safety and pathogen removal. All procedures have to be carried out according to good manufacturing practice (GMP) and using standard operating procedures (SOPs). Where cell culture manufacturers are required to maintain duplicated banks of cells to ensure product continuity, transgenic manufacturers would maintain sperm banks.

Animal husbandry. Regulations for the housing of transgenic animals will vary between different countries (see chapter 8). Veterinary health monitoring is required and, in addition, transgenic animals should be observed for any effects arising from recombinant protein expression. Generally, all animals will be kept under some type of containment regime, for example in double-fenced fields with each animal marked by identification tags and subject to strict accounting. Procedures for disposal of waste matter and cadavers to ensure suitable containment should be observed. EU rules for general animal husbandry (see section 8.2) also apply to transgenic herds or flocks.

Animals are generally raised according to their species' needs and requirements. Some restrictions to their freedom of movement may apply due to laws regarding genetically modified organisms (GMO) or the need for safekeeping from, for example, damage by animal rights activists. As a precaution, human access might be restricted.

2.4 Quality and safety of the product

In addition to the characterization steps detailed in section 2.2.5.1 for plant pharming and in 2.3.4.1 for animal pharming, several other factors must be addressed to ensure product quality and safety.

The health of production animals is important, not only to protect their own well-being but also to avoid possible transmission of zoonotic disease. As described above, regular monitoring by veterinarians is required. Strict precautions should also be taken to prevent contact with other farm animals or wild animals, or people or equipment that have been in recent contact with either. Concerns regarding transmission of prion diseases (BSE, scrapie) also mean that land used as animal pasture should not have had contact with other farm animals for several years. For this reason, some companies have chosen to raise animals in countries free of prion diseases, for example New Zealand. Alternatively, animals such as pigs and rabbits can be raised indoors in specific pathogen free facilities to minimize the risk of infectious diseases. Other precautions include exclusion of noxious agents, such as plant toxins or synthetic chemicals, for example pesticides.

In the case of plant pharming, great care has to be taken to avoid contamination with toxic or noxious soil constituents, chemicals present in the environment, and on the harvesting machinery, and chemicals like fertilizers or pesticides applied to crops and soil. Also soil bacteria, parasites, animal excreta and other unwanted substances preferably should be removed from the harvest before further processing can begin.

As with all biopharmaceuticals, production from transgenic animals and plants must comply with current FDA or EMEA guidelines and GMP. GMP compliance is a legal requirement (see chapter 8) and includes training of personnel, validation of procedures, equipment, materials and facilities, as well as SOPs. Production criteria must be defined at the outset, such as acceptance criteria for source material, product pooling, batch size and the product quality and purity required at various stages during purification to ensure product consistency. Throughout, documentation is essential and meticulous records must be kept of all activities, from the production of the DNA construct all the way to the final product.

The purified product should be characterized prior to final formulation in a manner similar to other biopharmaceuticals. In this regard the concept of the "well-characterized biologic" has been very important. This was defined in the US Federal register in 1996 as "a chemical entity whose identity, purity, impurities, potency, and quantity can be determined and controlled". Biopharmaceuticals of all types have sometimes encountered problems meeting this strict definition, and it is considerably more difficult than for chemically produced products. It is conceivable that this will be revised in the future. With regard to purity, acceptable low levels can be set for such contaminants as pathogens, host proteins, DNA and reagents

used in the purification process. However, it is more difficult to set acceptable standards for "identity" because very minor differences in protein glycosylation, folding and other post-translational modifications can alter bioactivity, efficacy and immunogenicity, possibly resulting in allergic or other adverse reactions in the patient.

Some early biological products are starting to come off patent, providing opportunities for cost-effective production in transgenic animals. These follow up products – so called "similar biological medicinal products" or "biosimilars" – will have to adhere to equally high quality and safety standards. The necessary legal framework has been established by regulatory authorities in the European Union. In the United States, the FDA are initiating discussions on this topic.

2.5 Choice of expression systems

Pharming has significantly extended the range of possible expression systems for biopharmaceutical proteins. Producers can choose between fermenter-grown transgenic mammalian cell cultures, transgenic bacteria or yeasts, transgenic plants or plant cell cultures and transgenic animals. The choice of production method is determined by several factors: the folding complexity of the protein, the nature and extent of post-translational processing required for protein activity, the quantity required and the value and the physiological function of the protein. Mammals are more appropriate than plants or microorganisms for the expression of proteins requir-

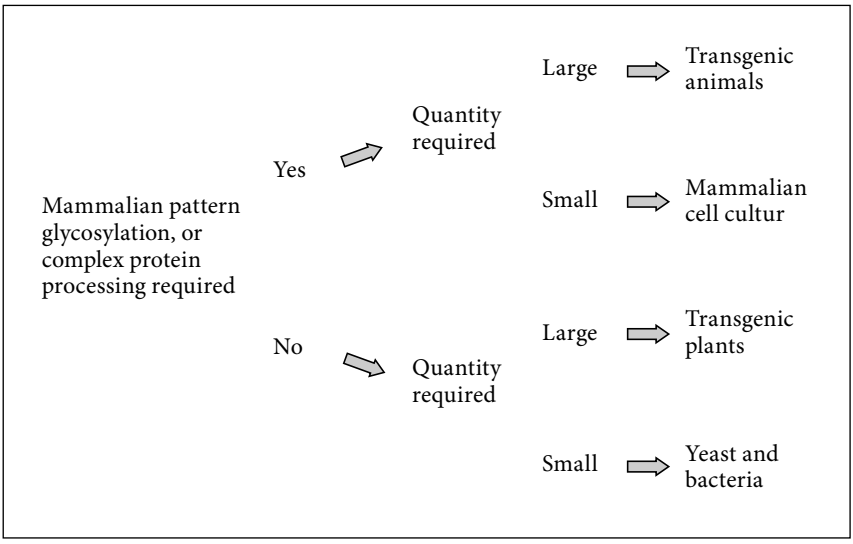


Figure 2.12: Choice of production platform for the manufacture of recombinant proteins

ing mammalian patterns of glycosylation, or complex post-translational processing for bioactivity. Because of the ease of scale-up, it is often argued that transgenic animals and plants are more suitable than cultured cells for proteins that are required in large volumes. This is summarized in figure 2.12 above.

2.6 References

Alan H, Christensen1and PH (1996) Quail1 Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Research 5(3):213–218

Azzoni AR, Kusnadi AR, Miranda EA, Nikolov ZL (2002) Recombinant aprotinin produced in transgenic corn seed: extraction and purification studies.Biotechnol Bioeng 5;80(3):268–276

Bardor M, Faveeuw C, Fitchette A-C, Gilbert D, Galas L, Trottein F, Faye L, Lerouge P (2003) Immunoreactivity in mammals of two typical plant glyco-epitopes, core (1,3)-fucose and core xylose. Glycobiology 13(6):427–434

Barta A, Sommergruber K, Thompson D, Hartmuth K, Matzke MA, Matzke AJM (1986) The expression of nopaline synthase – human growth hormone chimaeric gene in transformed tobacco and sunflower callus tissue. Plant Mol Biol 347–357

Beals TP, Goldberg RB (1997) A novel cell ablation strategy blocks tobacco anther dehiscence. Plant Cell 9:1527–1545

Bosch P, Pratt SL, Stice SL (2006) Isolation, characterization, gene modification, and nuclear reprogramming of porcine mesenchymal stem cells. Biol Reprod 74:46–57

Brigneti G, Voinnet O, Li W-X, Ji L-H, Ding S-W, Baulcombe DC (1998) Viral pathogenicity determinants are suppressors of transgene silencing in Nicotiana benthamiana. The EMBO Journal 17:6739–6746

Buchschacher GL Jr (2001) Introduction to retroviruses and retroviral vectors. Somat Cell Mol Genet 26:1–11

Burkhardt PK, Beyer P, Wünn J, Klöti A, Armstrong GA, Schledz M, von Lintig J, Potrykus I (1997) Transgenic rice (Oryza sativa) endosperm expressing daffodil (Narcissus pseudonarcissus) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. Plant J 11(5):1071–1078

Butaye KMJ, Cammue BPA, Delauré SL, De Bolle MFC (2005) Approaches to Minimize Variation of Transgene Expression in Plants. Molecular Breeding 16(1):79–91

Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996) Sheep cloned by nuclear transfer from a cultured cell line. Nature 380:64–66

Campbell KH, Alberio R, Choi I, Fisher P, Kelly RD, Lee JH, Maalouf W (2005) Cloning: eight years after Dolly. Reprod Domest Anim 40:256–268

Capecchi MR (2005) Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat Rev Genet 6:507–512

Clark AJ, Bessos H, Bishop JO, Brown P, Harris S, Lathe R, McClenaghan M, Prowse C, Simons JP, Whitelaw CBA, Wilmut I (1989) Bio/Technology 7:487–492

Clark AJ (1998) The mammary gland as a bioreactor: expression, processing, and production of recombinant proteins. J Mammary Gland Biol Neoplasia 3:337–350

Decker EL, Reski R (2007) Moss bioreactors producing improved biopharmaceuticals. Curr Opin Biotechnol 18(5):393–398

Dobrinski I (2005) Germ cell transplantation and testis tissue xenografting in domestic animals. Anim Reprod Sci 89:137–145

Drossart J (2004) Downstream processing of plant derived recombinant therapeutic proteins. In: Fischer R, Schillberg S (eds) Molecular farming. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Dyck MK, Gagne D, Ouellet M, Senechal JF, Belanger E, Lacroix D, Sirard MA, Pothier F (1999) Seminal vesicle production and secretion of growth hormone into seminal fluid. Nature Biotechnol 17:1087–1090

EMEA (2002) CPMP. Points to consider on quality aspects of medical producs containing active substances produced by stable transgene expression in higher plants. CPMP/BWP/764/02 (draft). The European Agency for the Evaluation of Medical Products (EMEA)

Ernst&Young (2003) Zeit der Bewährung – Deutscher Biotechnologiereport 2003

Fahrner RL, Knudsen CD, Basey CD (2001) Industrial purification of pharmaceutical antibodies: development, operation, and validation of chromatography processes. Biotechnol Genet Eng Rev 18:301–327

FDA (2002) Guidance for industry: Drugs, biologics, and medical devices derived from bioengineered plants for use in humans and animals (draft guidance). United States Food and Drug Administration

FDA (1982) Drug Bull 12(3):18–19

Gelvin SB (2003) Agrobacterium-Mediated Plant Transformation: the Biology behind the "Gene-Jockeying" Tool. Microbiol Mol Biol Rev 67(1):16–37

Giddings G, Allison A, Brooks D, Carter A (2000) Transgenic plants as factories for biopharmaceuticals. Nature Biotechnology 18:1151–1155

Gomord V, Chamberlain P, Jefferis R, Faye L (2005) Biopharmaceutical production in plants: problems, solutions and opportunities. Trends in Biotechnology 23(11):559–565

Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci U S A 77:7380–7384

Grill LK, Palmer KE, Pogue GP (2005) Use of Plant Viruses for Production of Plant-Derived Vaccines. Critical Reviews in Plant Sciences 24(4):309–323

Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W, Hasenfuss G (2006) Pluripotency of spermatogonial stem cells from adult mouse testis. Nature 440:1199–1203

Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680–683

Hitz C, Wurst W, Kühn R (2007) Conditional brain-specific knockdown of MAPK using Cre/loxP regulated RNA interference. Nucleic Acids Res 35:e90

Hofmann A, Kessler B, Ewerling S, Weppert M, Vogg B, Ludwig H, Stojkovic M, Boelhauve M, Brem G, Wolf E, Pfeifer A (2003) Efficient transgenesis in farm animals by lentiviral vectors. EMBO Rep 4:1054–1060

Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, Bailey M, Flynn P, Register J, Marshall L, Bond D, Kulisek E, Kusnadi A, Evangelista R, Nikolov Z, Wooge C, Mehigh RJ, Herman R, Kappel WK, Ritland D, Li CP, Howard JA (1997) Commercial production of avidin from transgenic maize: Characterization of transformant, production, processing, extraction and purification. Mol Breed 3:291–306

Hubner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, Wood J, Strauss JF 3rd, Boiani M and Schöler HR (2003) Derivation of oocytes from mouse embryonic stem cells. Science 300:1251–1256

Irion S, Luche H, Gadue P, Fehling HJ, Kennedy M, Keller G (2007) Identification and targeting of the ROSA26 locus in human embryonic stem cells. Nat Biotechnol 25:1477–1482

Irniger S, Sanfaçon H, Egli CM, Braus GH (1992) Different sequence elements are required for function of the cauliflower mosaic virus polyadenylation site in Saccharomyces cerevisiae compared with in plants. Mol Cell Biol 12(5):2322–2330

Jakobovits A, Amado RG, Yang X, Roskos L, Schwab G (2007) From XenoMouse technology to panitumumab, the first fully human antibody product from transgenic mice. Nat Biotechnol 25:1134–1143

Jenke AC, Stehle IM, Herrmann F, Eisenberger T, Baiker A, Bode J, Fackelmayer FO, Lipps HJ (2004) Nuclear scaffold/matrix attached region modules linked to a transcription unit are sufficient for replication and maintenance of a mammalian episome. Proc Natl Acad Sci U S A 101:11322–1137

Jung HS, Koo JK, Lee SJ, Park CI, Shin JY, Kim MH, Tan HK, Lim SM, Kim DI (2006) Characterization of human cytotoxic T lymphocyte-associated antigen 4-immunoglobulin (hCTLA4Ig) expressed in transgenic rice cell suspension cultures. Biotechnol Lett 28(24):2039–2048

Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, Baba S, Kato T, Kazuki Y, Toyokuni S, Toyoshima M, Niwa O, Oshimura M, Heike T, Nakahata T, Ishino F, Ogura A, Shinohara T (2004) Generation of pluripotent stem cells from neonatal mouse testis. Cell 119:1001–1012

Kanatsu-Shinohara M, Ikawa M, Takehashi M, Ogonuki N, Miki H, Inoue K, Kazuki Y, Lee J, Toyokuni S, Oshimura M, Ogura A, Shinohara T (2006) Production of knockout mice by random or targeted mutagenesis in spermatogonial stem cells. Proc Natl Acad Sci U S A 103:8018–8023

Kato Y, Imabayashi H, Mori T, Tani T, Taniguchi M, Higashi M, Matsumoto M, Umezawa A, Tsunoda Y (2004) Nuclear transfer of adult bone marrow mesenchymal stem cells: developmental totipotency of tissue-specific stem cells from an adult mammal. Biol Reprod 70:415–8

Katsnelson A, Ransom J, Vermij P, Waltz E (2006) News In Brief, Nature Biotechnology 24:233–234

Kerr DE, Liang F, Bondioli KR, Zhao H, Kreibich G, Wall RJ, Sun TT (1998) The bladder as a bioreactor: Urothelium production and secretion of growth hormone into urine. Nature Biotechnol 16:75–79

Kim TG, Baek MY, Lee EK, Kwon TH, Yang MS (2008) Expression of human growth hormone in transgenic rice cell suspension culture. Plant Cell Rep 27(5):885–891

Kioussis D, Festenstein R (1997) Locus control regions: overcoming heterochromatin-induced gene inactivation in mammals. Curr Opin Genet Dev 7:614–619

Klein TM, Arentzen R, Lewis PA, Fitzpatrick-McElligott S (1992) Transformation of microbes, plants and animals by particle bombardment. Biotechnology 3:286–291

Knäblein J (2005) Plant-based expression of biopharmaceuticals. In: Meyers R (ed) Encyclopidia of molecular cell biology and molecular medicine. 2nd Edition. Volume 10. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Kumria R, Waie B, Rajam MV (2001)Plant regeneration from transformed embryogenic callus of an elite indica rice via Agrobacterium. Plant Cell, Tissue and Organ Culture 67(1):63–71

Kuroiwa Y, Kasinathan P, Choi YJ, Naeem R, Tomizuka K, Sullivan EJ, Knott JG, Duteau A, Goldsby RA, Osborne BA, Ishida I, Robl JM (2002) Cloned transchromosomic calves producing human immunoglobulin. Nat Biotechnol 20:889–894

Kuvshinov V, Anissimov A, Yahya BM (2004) Barnase gene inserted in the intron of GUS – a model for controlling transgene flow in host plants. Plant Science 167(1):173–182

de Laat W, Grosveld F (2003) Spatial organization of gene expression: the active chromatin hub. Chromosome Res 11:447–59

Labosky PA, Barlow DP, Hogan BL (1994) Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. Development 120:3197–3204

Lal P, Ramachandran VG, Goyal R, Sharma R (2007) Edible vaccines: current status and future. J Med Microbiol 25(2):93–102 Review

Lavitrano M, Busnelli M, Cerrito MG, Giovannoni R, Manzini S, Vargiolu A (2006) Sperm-mediated gene transfer. Reprod Fertil Dev 18:19–23

Lee SJ, Park CI, Park MY, Jung HS, Ryu WS, Lim SM, Tan HK, Kwon TH, Yang MS, Kim DI (2007) Production and characterization of human CTLA4Ig expressed in transgenic rice cell suspension cultures. Protein Expr Purif 2:293–302

Lillico SG, Sherman A, McGrew MJ, Robertson CD, Smith J, Haslam C, Barnard P, Radcliffe PA, Mitrophanous KA, Elliot EA, Sang HM (2007) Oviduct-specific expression of two therapeutic proteins in transgenic hens. Proc Natl Acad Sci U S A 104:1771–1776

Love J, Gribbin C, Mather C, Sang H (1994) Transgenic birds by DNA microinjection. Biotechnology (N Y) 12:60–63

Ma JK, Drake PM, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4(10):794–805

Maga EA, Walker RL, Anderson GB, Murray JD (2006) Consumption of milk from transgenic goats expressing human lysozyme in the mammary gland results in the modulation of intestinal microflora. Transgenic Res 15:515–519

Manzini S, Vargiolu A, Stehle IM, Bacci ML, Cerrito MG, Giovannoni R, Zannoni A, Bianco MR, Forni M, Donini P, Papa M, Lipps HJ, Lavitrano M (2006) Genetically modified pigs produced with a nonviral episomal vector. Proc Natl Acad Sci U S A 103:17672–7677

Marshall B (2007) PMPs in clinical trials and advanced PMIPs - Research findings first presented at FinMed 2006, on 30th March 2006 - table last updated Oct.07, http://www.molecularfarming.com/PMPs-and-PMIPs.html (July, 2008)

Masarik M, Kizek R, Kramer KJ, Bilova S, Brazdova M, Vacek J, Bailey M, Jelen F, Howard JA (2003) Application of avidin-biotin technology and adsorptive transfer stripping square-wave voltammetry for detection of DNA hybridization and avidin in transgenic avidin maize. Anal Chem 75:2663–2669

Mason HS, Lam DM, Arntzen CJ (1992) Expression of hepatitis B surface antigen in ransgenic plants. Proc Natl Acad Sci USA 89:1174–11749

Matsumoto S, Ikura K, Ueda M (1995) Plant Molecular Biology 27:1163–1172

McCreath KJ, Howcroft J, Campbell KHS, Colman A, Schnieke AE, Kind AJ (2000) Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 405:1066–1069

Meissner A, Wernig M, Jaenisch R (2007) Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. Nat Biotechnol 25:1177–1181

Moreira PN, Pozueta J, Pérez-Crespo M, Valdivieso F, Gutiérrez-Adán A, Montoliu L (2007) Improving the generation of genomic-type transgenic mice by ICSI. Transgenic Res 16:163–168

Nayernia K, Nolte J, Michelmann HW, Lee JH, Rathsack K, Drusenheimer N, Dev A, Wulf G, Ehrmann IE, Elliott DJ, Okpanyi V, Zechner U, Haaf T, Meinhardt A, Engel W (2006a) In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. Dev Cell 11:125–132

Nayernia K, Lee JH, Drusenheimer N, Nolte J, Wulf G, Dressel R, Gromoll J, Engel W (2006b) Derivation of male germ cells from bone marrow stem cells. Lab Invest 86:654–663

Nykiforuk CL, Boothe JG, Murray EW, Keon RG, Goren HJ, Markley NA, Moloney MM (2006) Transgenic expression and recovery of biologically active recombinant human insulin from Arabidopsis thaliana seeds. Plant Biotechnology Journal 4(1):77–85

Oback B, Wells DN (2007) Donor cell differentiation, reprogramming, and cloning efficiency: Elusive or illusive correlation? Mol Reprod Dev 74:646–654

Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. Nature 448:313–317

Petitte JN, Liu G, Yang Z (2004) Avian pluripotent stem cells. Mech Dev 121:1159–1168

Pfeifer A (2004) Lentiviral transgenesis. Transgenic Res 13:513–522

Prelle K, Zink N, Wolf E (2002) Pluripotent stem cells-model of embryonic development, tool for gene targeting, and basis of cell therapy. Anat Histol Embryol 31:169–186
Pujol M, Gavilondo J, Ayala M, Rodríguez M, González EM, Pérez L (2007) Fighting cancer with plant-expressed pharmaceuticals. Trends in Biotechnology Vol 25, Issue 10:455–459
Rapp JC, Harvey AJ, Speksnijder GL, Hu W, Ivarie R (2003) Biologically active human interferon alpha-2b produced in the egg white of transgenic hens. Transgenic Res 12:569–575
Sanford JC, Klein TM, Wolf ED, Allen N (1987) Delivery of substances into cells and tissues using a particle bombardment process. Journal of Particulate Science and Technology 6:559–563
Sanford JC (1988) The Biolistic Process. Trends in Biotechnology 6:299–302
Sato M (2006) Direct gene delivery to urine testis as a possible means of transfection of mature sperm and epithelial cells lining epididymal ducts. Reprod Med & Biol 5:1–7
Sauter A (2005) Grüne Gentechnik – transgene Pflanzen der 2. und 3. Generation. Arbeitsbericht des Büros für Technikfolgen-Abschätzung beim Deutschen Bundestag, No. 104
Schlappi M, Hohn B (1992) Competence of Immature Maize Embryos for Agrobacterium-Mediated Gene Transfer .The Plant Cell, 4(1):7–16
Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KHS (1997) Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 278:2130–2133
Shadwick FS, Doran PM (2007) Infection, propagation, distribution and stability of plant virus in hairy root cultures. Journal of Biotechnology 131(3):318–329
Shadwick FS, Doran PM (2004) Foreign Protein expression using plant cell suspention and hairy root cultures. In: Fischer R, Schillberg S (eds) Molecular farming. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim
Sharp JM, Doran PM (2001) Strategies for enhancing monoclonal antibody accumulation in plant cell and organ cultures. Biotechnol Prog 17(6):979–992
Stöger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P, Fischer R (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. Plant Mol Biol 42(4):583–90
Swanson ME, Martin MJ, O'Donnell JK, Hoover K, Lago W, Huntress V, Parsons CT, Pinkert CA, Pilder S, Logan JS (1992) Production of functional human hemoglobin in transgenic swine. Biotechnology (N Y) 10:557–559
Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–72
Trombetta ES, Parodi AJ (2003) Quality control and protein folding in the secretory pathway. Annu Rev Cell Dev Biol 19:649–676
Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: Host systems and expression technology. Trends Biotechnol 21 (12):570–578
Vajta G, Gjerris M (2006) Science and technology of farm animal cloning: state of the art. Anim Reprod Sci 92:211–30
Wakayama T, Perry AC, Zuccotti M, Johnson KR and Yanagimachi R (1998) Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394:369–374
Walsh G (2006) Biopharmaceutical benchmarks 2006. Nat Biotechnol 7:769–776
Walsh G (2003) Biopharmaceuticals: Biochemistry and Biotechnology. Wiley
Wells DN (2005) Animal cloning: problems and prospects. Rev Sci Tech 24:251–264

Whitelaw CB, Radcliffe PA, Ritchie WA, Carlisle A, Ellard FM, Pena RN, Rowe J, Clark AJ, King TJ, Mitrophanous KA (2004) Efficient generation of transgenic pigs using equine infectious anaemia virus (EIAV) derived vector. FEBS Lett 571:233–236

Willadsen SM (1986) Nuclear transplantation in sheep embryos. Nature 320:63–65

Wilmut I, Schnieke A, McWhir J, Kind AJ, Campbell KHS (1997) Viable offspring derived from foetal and adult mammalian cells. Nature 385:810–813

Wobus AM, Boheler KR (2005) Embryonic stem cells: prospects for developmental biology and cell therapy. Physiol Rev 85:635–678

Wright G, Carver A, Cottom D, Reeves D, Scott A, Simons P, Wilmut I, Garner I and Colman A (1991) High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. Biotechnology 9:830–834

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920

Zhu L, van de Lavoir MC, Albanese J, Beenhouwer DO, Cardarelli PM, Cuison S, Deng DF, Deshpande S, Diamond JH, Green L, Halk EL, Heyer BS, Kay RM, Kerchner A, Leighton PA, Mather CM, Morrison SL, Nikolov ZL, Passmore DB, Pradas-Monne A, Preston BT, Rangan VS, Shi M, Srinivasan M, White SG, Winters-Digiacinto P, Wong S, Zhou W, Etches RJ (2005) Production of human monoclonal antibody in eggs of chimeric chickens. Nat Biotechnol 23:1159–1169