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—Review—

# Basics and Applications of Gene Transfer in Animal Cells and Tissues for Production and Therapeutics

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## Reappraisal of Transgenic Nomenclature

The possibility that animal cells could be altered in selected and intended ways by introducing foreign DNA into them has long enchanted scientists. The experimental protocols of gene transfer now become feasible both *in vitro* in cultured cells and *in vivo* in tissues of living animals. A number of recent reviews pay attention primarily to the means of gene transfer, not to cells or animals themselves subjected to gene transfer, and therefore adequate scientific terms to describe such cells and animals are definitely lacking. What have been proposed for defining animals are only "transgenic" and "nontransgenic" in the classical nomenclature. In cell culture systems, they are sometimes called stable transformants or simply those transiently expressing foreign genes, irrespective of the origin of the cells. Because many exceptions have emerged from drastic progress in genetic engineering today in farm and experimental animals, the classical scientific terms and definitions are outdated, and no longer sufficient.

In the present review, therefore, a new classification is presented in Fig. 1 to define various types of "transgenic" cells and animals including humans to which foreign genes are transferred, in conjunction with the possible application of these cells and animals for experimental and therapeutic purposes. Figure 1 also emphasizes how some types of transgenic cells and animals could be converted to others, rather than how they are made by using certain gene transfer techniques. The classical term for transgenic animals used herein is "genuine transgenic animals" in which foreign genes are experimentally introduced and integrated in the genome of germ cells so that the genes can be transmitted to progeny. Other types of cells and animals, irrespective of the status of the transgenes, are also classified under different transgenic names.

Among these, of course, the most notable is cultured somatotransgenic cells with the "Dolly pathway", in which somatic cells are utilized to obtain animal clones as seen in the cloned sheep [131]. Although not as popular as the Dolly pathway, the way in which embryo-derived cells totipotent for nuclear transfer are used to obtain sheep clones [15] is also of substantial importance as a new method for making genuine transgenic animals. The significance of these two types of cells and pathways is obvious. Embryonic stem (ES) cells are not necessarily the only source of knock-out or knock-in farm animals, but any germ or somatic cells could be applicable to gene targeting procedures so long as they are totipotent for nuclear transfer. In addition to the transgenic cells and animals deduced from the above findings, a group of transfectgenic cells and animals designated as "transfectgenic" have been presented here, though they may be unfamiliar to readers. Most likely consequences of gene transfer attempts would be the production of such transfectgenic cells and animals that carry transgenes only with an episomal form. As a result, transgenes disappear as time goes by. Generally speaking, the production of transfectgenic cells and animals may be easier than other types of transgenic cells and animals, but they could serve not only as experimental models for basic studies but also as important sources of food and biomedical protein.

It is beyond the scope of this review to cover all aspects of these cells and animals and gene transfer methods involved. Instead, emphasis is placed upon,

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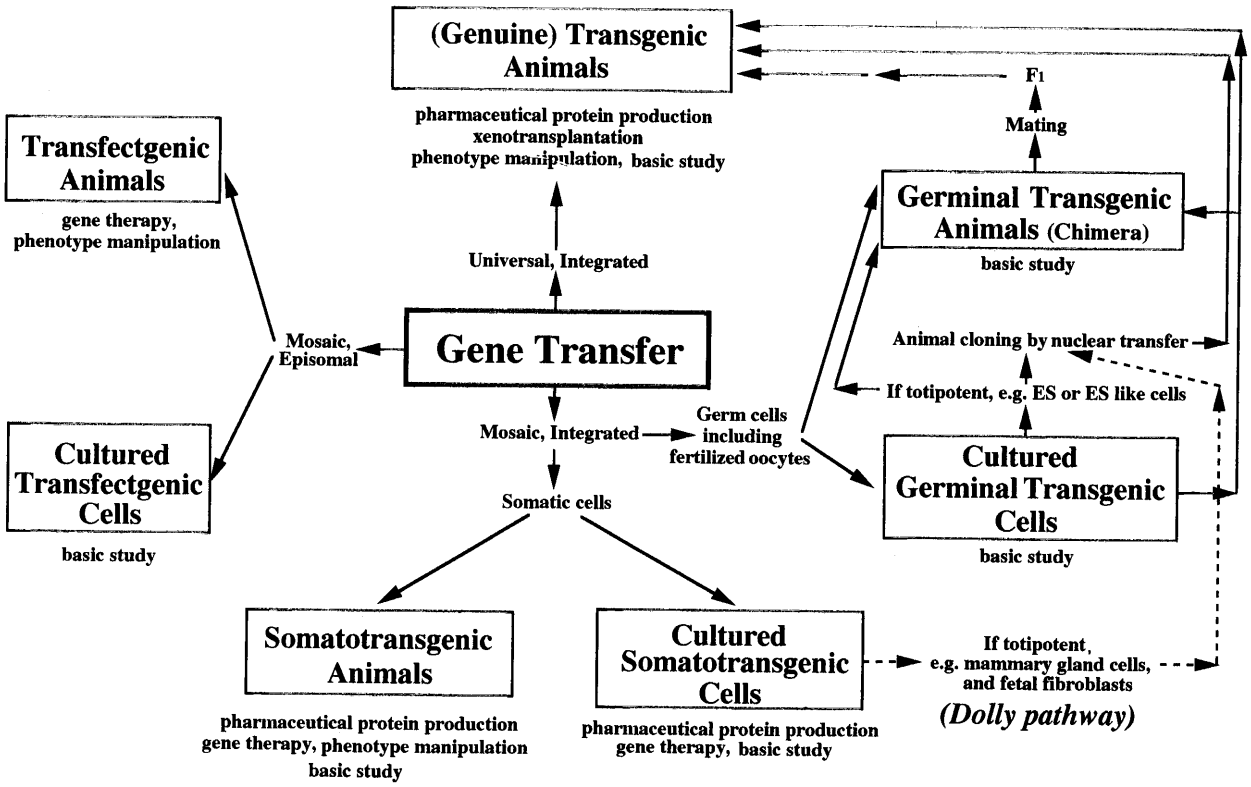


**Fig. 1.** Classification of various transgenic cells and animals including humans to which foreign genes are transferred, and possible conversions from some types of animals and cells to others in conjunction with possible applications of these cells and animals for experimental and therapeutic purposes. The classical term transgenic animals herein is designated as "(genuine) transgenic animals" in which foreign genes are experimentally introduced and integrated in the genome of germ cells so that the genes can be transmitted to progeny. Other types of cells and animals, irrespective of the status of transgenes, are also classified under different transgenic names.

(1) basics and principles of general gene transfer procedures, (2) gene transfer and related techniques to make genuine transgenic animals via cultured germinal transgenic cells, and (3) transfectgenic, and somatotransgenic animals with respect to their possible applications to experimental and therapeutic purposes.

## Basics and Principles of General Gene Transfer Techniques

Table 1 summarizes currently available gene transfer methods in cultured cells and tissues of living animals. Some are applicable to both *in vitro* and *in vivo* situations whereas others are specifically employed only in either system.

In early studies, experimental attempts to transfer foreign genes have started in the cell culture system *in vitro*. The major obstacle to the uptake of DNA by cells is that in water solution the DNA molecule has a negative electrical charge, which results in repelling the cell membranes that are also negatively charged. In order to attain smooth and efficient transfer of foreign DNA into animal cells, the negative electrical charge may have to be somehow neutralized. Such neutralization was attempted by means of a positively charged organic polymer called DEAE-dextran [69], calcium phosphate [39] and polybrene [17]. Although the mechanism remains obscure, it is believed that the transfected DNA enters the cytoplasm of the cell by endocytosis and is transferred to the nucleus. Neutralization can also be achieved by a different approach in which a DNA-containing solution is held within a double layer of lipid molecules. DNA transfection with such double layer liposomes or cation liposomes, called lipofection, has been intensively studied as a delivery vehicle [30, 67].

In other means of foreign gene transfer *in vitro*, little attention has been paid to neutralization. Instead, different strategies have been employed. They include protoplast fusion which utilizes membrane fusion with protoplasts carrying a large number of plasmid copies

**Table 1.** Methods of gene transfer in cultured animal cells (*in vitro*) and tissues of living animals (*in vivo*)

| *In Vitro* | *In Vivo* |
|---|---|
| Biological Means | |
| Viral vectors | Viral vectors |
| Receptor mediation | Receptor mediation |
| Protoplast fusion | |
| Chemical Means | |
| Lipofection | Lipofection |
| Calcium phosphate precipitation | |
| DEAE dextran mediation | |
| Polybrene mediation | |
| Physical Means | |
| Gene gun | Gene gun |
| Electroporation | Electroporation |
| Laserporation | Laserporation |
| Pricking | Direct injection |
| Microinjection | |

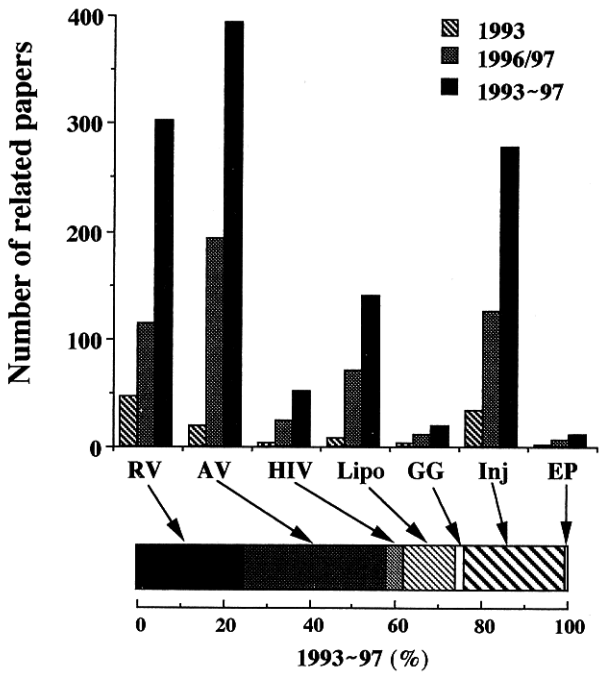


**Fig. 2.** The number of papers encountered in the search in MEDLINE from 1993 through 1997 (August) under the phrases *in vivo* (or ex vivo) gene transfer or gene therapy. The proportion of each entry from 1993 to 1997 is also shown below the actual numbers of papers. The actual proportion should be more favour to viral vector methods as no vectors based on adenoassoicated viruses, herpesviruses, alphaviruses or poxviruses are included. Abbreviations used: RV, retroviral vectors; AV, adenoviral vectors ; HIV, human immunodeficiency viral vectors; Lipo, lipofection ; GG, gene gun ; Inj, direct DNA injection; EP, electroporation. Adapted from Muramatsu *et al.* [78].

[107], electroporation and laser optoporation, both of which are based on the formation of nanometer-sized pores by electric pulses [136] and laser beams [94] respectively, and microinjection which is attained by direct DNA injection into nuclei with a fine-drawn pipette [16].

In contrast to *the in vitro* situation, *in vivo* gene transfer has hitherto largely relies on viral vectors. In the long history of the evolution of life, viruses have developed specific mechanisms for cell attachment, penetration and replication so that they become natural transporters of genes to animal cells. In Fig. 2, the proportion of several *in vivo* methods used in recent years is shown, indicating that viral vector methods account for the majority, more than 60%, of the total number of papers concerning *in vivo* gene transfer [78]. But there are serious concerns about the use of viral vectors especially when gene therapy for humans is to be attempted. Retroviral vectors, for instance, usually suffer from low titers, oncogenic potential, and the requirement of active cell division for integration. Although adenoviral vectors can be recovered in high titers and could transfer genes to both dividing and nondividing cells, host immunogenecity prevents their repeated use [32].

For researchers, an easy, safe, non-toxic, and possibly efficient delivery of genes to a specified target tissue has been an attractive issue. Hence, the above limitations of the use of viral vectors give an impetus to alternative nonviral means of gene transfer. As shown in Fig. 2, the number of papers concerning nonviral gene transfer methods gradually increased between 1993 and 1996/7. Particularly notable is the rapid increase in lipofection and direct DNA injection. The advantages and disadvantages of these popular nonviral methods have been discussed elsewhere [30, 109]. Apart from these two popular nonviral methods, electroporation (EP) is also found to be efficient *in vivo* in tissues of living animals [23, 46, 126]. EP is a well-known technique for introducing DNA or chemical reagents into animal cells, plant cells and bacteria in culture by using pulsed electric fields [2, 21, 33, 85]. Although EP accounts for only about 1% of papers on *in vivo* gene transfer and gene therapy (Fig. 2), there are good reasons to believe that this is going to be more widely used. EP has a variety of advantages over other nonviral as well as viral vectors since all tissues and cells in theory could become a target, handling is easy and quickly completed within a matter of second, repeated administration of DNA is possible, no immunogenicity is expected, no DNA size constraints are imposed, and no specialized process for DNA construction is required. The *in vivo* gene transfer

technique, particularly by using *in vivo* gene EP, will be discussed in more detail later, in the section concerning transfectgenic and somatotransgenic animals.

## Gene Transfer and Related Techniques to Make Genuine Transgenic Animals with Cultured Germinal Transgenic Cells

*(1) Gene transfer methods*

Although to date a variety of genuine transgenic farm animals have been successfully produced, cattle are the most difficult animal species to use for this purpose. Eyestone [28] has suggested that the production of transgenic cattle presents a unique set of challenges relative to other species. Those problems are: low frequency of generating transgenic offspring from microinjected zygotes, poor embryo and fetal survival, low transgene integration frequency, and many recipient herds needed. Despite these obstacles, enormous amounts of milk proteins produced per head have kept attracting scientists to the challenge of transgenic cattle production. In the following sections, the description is mainly focused on gene transfer and related techniques applied to bovine germ cells. Clearly the step at gene transfer is of crucial importance in overall efficiency in producing transgenic cattle. As argued by Strijker *et al.* [116], one would not doubt that one of the most promising applications of transgenic cattle is to produce pharmaceutical proteins that are relatively inexpensive, but required in large quantity, such as human lactoferrin.

Currently applicable methods for transferring genes to bovine germ cells include microinjection (into pronuclei or cytoplasm), microparticle bombardment, retrovirus vectors, sperm-mediation, and nuclear transfer. Of these, by far the most widely employed method is the microinjection of foreign DNA into the pronucleus of fertilized oocytes, mainly because the method has been successfully and routinely used in other animal species. But in cattle, the integration of microinjected transgenes occurs much less frequently than in other animal species. According to Eyestone [28], the proportion of transgenic cattle to the total zygotes injected is only 0.09% compared with 0.8 to 1.2% for sheep, goats and pigs. Such poor integration frequency could possibly be mitigated by co-injecting carrier genomic DNA of bovine origin since Akasaka *et al.* [1] reported that the co-introduction of a transgene with carrier DNA fragmented by restriction enzyme digestion resulted in accelerated integration of a reporter transgene into the genome.

In addition to the poor integration frequency, a reduced developmental potential of zygotes after microinjection of transgenes has been recorded in cattle [51] as well as goats [110] and sheep [103]. Handling of oocytes mimicking gene transfer manipulation conditions as in mice was not in itself responsible for such decreased developmental potential. DNA microinjection conducted at the mid to late pronuclear stages did not decrease DNA detection frequency, but did decrease embryo development [59, 60, 97, 98].

Because of the lipid content and opacity of the bovine oocyte, DNA microinjection into pronuclei in cattle is more difficult than in mice. To make it easier, cytoplasmic injection of DNA has been tested. Powell *et al.* [100] found, for example, in cattle, sheep and pigs that the frequency of integrating DNA into the genome by cytoplasmic injection was lower than that by pronuclear injection, but treating DNA with polylysine prior to the cytoplasmic injection restored the poor integration frequency to a level as high as that attained in the pronuclear DNA injection [92]. The presence of polylysine with foreign DNA appeared to alter the *in vitro* activities of restriction endonuclease and DNA ligase on foreign DNA, and to protect DNA injected into cytoplasm by forming a complex of DNA/polylysine which, in turn, may act as a better substrate for transgenesis. If this were also applicable to farm livestock including cattle, the cytoplasmic injection would considerably alleviate the difficulty in pronuclear injection of DNA in this animal species.

Among the DNA transfer techniques not relying on direct injection with a fine-drawn pipette, the most successful approach has been the use of viral vectors. By means of retroviral vectors, genes have been effectively transferred into the embryos of mice [29, 50, 124, 132], pigs [54], chickens [8, 105] and zebrafish [65]. The first successful use of retroviral vectors in cattle was reported by Kim *et al.* [57], who showed that not only the trophectoderm but also the inner cell mass retained the integrated transgene. As a variant of the retroviral vector technique, Haskell and Bowen [43] microinjected retrovirus producer cells in the perivitelline space of one- to four-cell bovine embryos, and obtained transgenic fetuses that had identical patterns of integration in several tissues within each fetus.

Adenoviral vectors represent an alternative choice for virus-mediated transgenesis. It is generally believed that adenoviral vectors can deliver transgenes for non-dividing differentiated cells without integrating transgenes so that the transgene delivered to the cell by this vector is believed to exist as an episomal form in the nucleus. But a recent work by Tsukui *et al.* [123] demonstrated

that a replication-defective adenoviral vector did, in fact support transgenesis in mice, resulting in about 10% transgenic pups when infected into zona-free oocytes. It appears that the integration frequency obtained by this method is higher than would be expected in fertilized oocytes cultured *in vitro*, possibly due to their large size, and therefore the large surface area relative to any other type of cell. Although the exact mechanism of this high integration frequency is unclarified, the adenoviral vector method may also provide a useful source of viral vectors for transgenesis.

Embryonic stem (ES) cells are now routinely used in gene transfer and targeting work in mice. The use of these cells facilitates making almost any desired change in the genome of the mouse [104]. ES cells are initially isolated from the inner cell mass of the blastocyst and grown in culture. In attempting to derive ES cells from cattle, problems arise because exactly analogous stages do not exist in embryos of mice and ungulates owing to differences in their embryonic development. The inner cell mass of the bovine embryo undergoes little development relative to the trophectoderm for several days in the elongating blastocyst, which differs noticeably from the way development of the inner cell mass occurs in the mouse. Nevertheless, considerable progress has been made in recent years in establishing apparently stable ES-like stem cell lines from cattle embryos [113]. Perhaps the most serious disadvantage in using ES cells for the transfer of foreign DNA in cattle is the fact that the calves born after the injection of ES cells will be chimeras. It takes at least 6 years before homozygote calves are born with transgenes in cattle [130].

Totipotent primordial germ (PG) cells offers better opportunities than ES cells for making transgenic animals if they can be isolated and maintained in culture. The advantage of using such cells in place of ES cells is obvious only some ES cells could differentiate to germ cells whereas in principle all PG cells will form gonads, leading to the generation of spermatogenic cells or oocytes. The first report on PG cells came from a mouse study by Resnick *et al.* [102], opening up a new route for transgenic technology in cattle as well as in other livestock. Cherny and Merei [19] reported the establishment of totipotent bovine PG cell-derived cell lines maintained in long-term culture. Lavoir *et al.* [63,64] also reported the isolation and identification of female germ cells from bovine gonadal cell suspensions between days 35 and 130 of gestation. As reviewed by Cherny *et al.* [20], whether these cells are truly able to differentiate to germ cells with integrated transgenes remains to be examined.

The nuclear transfer technique, if combined with derivation of the afore mentioned ES cells or totipotent germ cells, could provide a powerful tool for producing transgenic animals in important farm livestock. This possibility has now greatly increased since the reports of cloning lambs [15] and calves [114] by the nuclear transfer technique.

Indeed, Krisher *et al.* [61] claimed that bovine embryos that had been microinjected with DNA could be efficiently utilized as donor embryos in nuclear transfer. Germ cell nuclei are alternatives as a nucleus donor for enucleated oocytes since this manipulation was found to be possible in mice [56]. Moreover, the recent report on the cloned sheep, Dolly, has entirely changed our belief in such a way that not only germ cells but also somatic cells that are fully differentiated could also serve as totipotent donor cells for nuclear transfer procedures [131]. It may be that the cell type, whether germ or somatic, does not matter very much so long as recipient oocytes are subjected to proper reprogramming procedures. Unfortunately the birth rate is quite poor by the currently available nuclear transfer techniques. This should result in extremely low production rates of genuine transgenic cattle. Provided that the nuclear transfer provides a high yield of cloned calves, the only remaining obstacle is how to select the nuclear-donor cells that carry transgenes of interest. This problem would be mitigated by using reporter gene expression as a marker of donor embryos in nuclear transfer. The day may not be far away when such techniques are routinely used in practice.

A very simple and convenient method, and therefore of great interest, came from the sperm-mediated transgenesis technology. The first indication goes back more than 20 years [11]. Since then several reports have indicated that this might be a way of introducing foreign genes into the germ cells of animals [18, 36, 47, 62], but Brinster *et al.* [13] have expressed doubt about this because studies on sperm-mediated gene transfer in their own and several other laboratories failed to produce even a single transgenic mouse in more than 1,300 births. So far it has become gradually evident that the sperm of a variety of animal species could indeed bind foreign DNA [35, 47, 62, 96]. The question is whether or not the DNA adsorbed onto the surface of sperm can be efficiently delivered to the pronucleus where the foreign DNA is integrated even though at a rare frequency. Therefore, the most likely causes of discrepancy found in the previous reports may be the extremely low frequency at which sperm binds the DNA and helps the integration processes. Unless a new means to improve

such low frequency of DNA binding and integration is found, the future of the sperm-mediated transgenesis technique remains elusive.

If foreign DNA could be transferred to spermatogenic cells, the differentiated sperms could eventually be a vector carrying transgenes as well as genomic DNA like those supposed to be involved in the sperm-mediated transgenesis. Since at present no good *in vitro* culture method is available for spermatogenic cells to fully differentiate into mature sperm, approaches to this spermatogenic-cell mediated transgenesis appeared to be virtually closed. But the recent finding that a foreign gene could be transferred to mouse spermatogenic cells *in vivo* [80] indicated the possibility of this approach. Later, it was found that the gene expression in the testis of living mice lasted for at least 4 weeks [82] and even up to 8 weeks [unpublished results], which is long enough for a spermatogonium to differentiate into a mature sperm stage. Like the sperm-mediated transgenesis, improvement of gene integration frequency and *in vivo* enrichment of such transgene-carrying spermatogenic cells are also the major subjects related to spermatogenic cell-mediated transgenesis. Development of new techniques that open up the seemingly narrow path is awaited.

*(2) Screening methods for genuine transgenic animals at preimplantation stages*

One of the prohibitive costs in making transgenic cattle is that of maintaining recipient cattle bearing non-transgenic pregnancies. To avoid this, reliable and convenient embryo selection strategy should be devised. In principle, either DNA or protein analysis could be utilized. So far, however, the DNA-based analysis has exclusively been studied. Although some modifications were made to avoid picking up false positive signals, DNA-based PCR analyses resulted in diagnosing a dominant portion of non-transgenic embryos as being transgenic [28]. The source of DNA causing these false positive signals is unknown. Some of this DNA may exist on the blastomere cell membranes or on the zona pellucida. The reported proportions of transgene positive bovine morulae or blastocysts detected by PCR analysis ranged from 21 to 85% (see Table 2). In contrast to these substantially high rates of detecting false positives, Seo *et al.* [111] reported that the non-integrated injected DNA was almost completely eliminated by their modified PCR procedure, but as the procedure is time-consuming and complicated, its use might be impractical.

With respect to the selection efficiency, simplicity and short time period required, protein-based analysis might be a more attractive alternative, although few attempts have been made hitherto. Takeda and Toyoda [117] showed that the lacZ reporter gene expression could be detectable by X-gal staining in developing mouse oocytes. Likewise in bovine embryos, Nakamura *et al.* [83] demonstrated that the detection of the bacterial lacZ gene expression was possible after microinjecting the reporter gene driven by the SV40 promoter. But the great disadvantage of the X-gal staining method was that developing embryos have to be fixed prior to histochemical staining for the detection of lacZ expression, and therefore they are no longer transplantable after the detection of the lacZ gene expression.

Thompson *et al.* [119] have eliminated this shortcoming by using the live detection and screening method based on the bioluminescence generated from the firefly luciferase gene expression in the mouse oocyte. Whether or not the same bioluminescence screening method could be applied to bovine oocytes was tested in bovine oocytes. It was demonstrated that, as shown in Fig. 3, clear bioluminescence was detected in 4- to 16-cell bovine embryos after microinjecting with the firefly luciferase followed by single photon imaging [77]. The possible toxicity of luciferin, substrate for the bioluminescence reaction, to the embryonic development would be negligible since the detection can be completed within 10 min. Such a selection strategy may be equivalent to or more efficient than those reported for the PCR-based selection method [unpublished results]. With this technology, Nakamura *et al.* [84] found that the hybrid promoter of Rous sarcoma virus long terminal repeat and chicken $\beta$-actin gene conferred strong

**Table 2.** Proportions of transgenes detected by either PCR analysis or luciferase bioluminescence in preimplantation embryos

| Species | Method | Transgene positive (%) | References |
|---|---|---|---|
| Mouse | PCR | 36 (morula) | Ninomiya *et al.* [88] |
| Mouse | PCR | 44 (morula) | Burdon and Wall [14] |
| " | PCR | 26 (blastocyst) | " |
| Mouse | PCR | 40 (morula) | Page *et al.* [92] |
| Bovine | PCR | 54 (blastocyst) | Behboodi *et al.* [4] |
| Bovine | PCR | 60 (blastocyst) | Horvat *et al.* [48] |
| Bovine | PCR | 21 (blastocyst) | Bowen *et al.* [10] |
| Bovine | PCR | 85 (blastocyst) | Krisher *et al.* [59] |
| Bovine | luciferase | 21 (morula/ blastocyst) | Muramatsu *et al.* [unpublished] |

Adapted from Muramatsu and Nakamura [77].

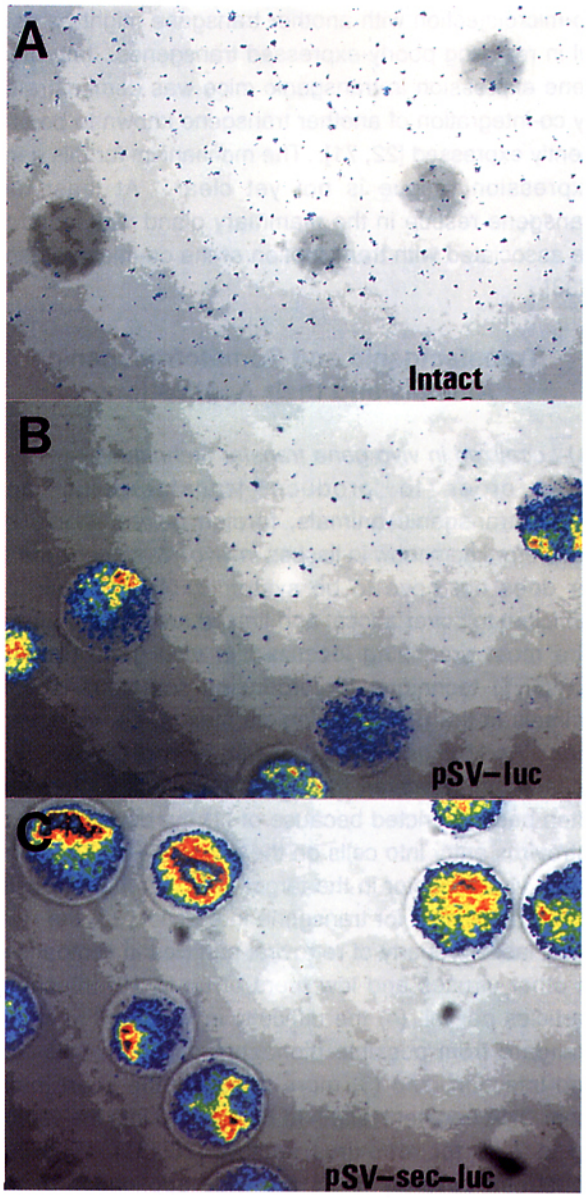


**Fig. 3.** Superimposed images of light and bioluminescence of fertilized bovine oocytes microinjected with TE buffer only (Intact: A), fertilized bovine oocytes microinjected with pSVluc (B), and microinjected with pSVsecluc (C). Bioluminescence imaging analysis was done at 2 days after microinjection by making a cumulative count of photons for 10 min. For the bioluminescence reaction, 100 $\mu$l of 500 $\mu$M luciferin solution was added to the culture medium. The color code at the right side indicates the intensity of the accumulated photons, ranging from low (blue) to high (red). No luciferase expression was detected in the intact oocytes (× 200). Adapted from Muramatsu and Nakamura [77].

transcriptional activity as shown by bioluminescence, and therefore would be suitable for embryo selection in combination with the firefly luciferase reporter gene. As might be suspected, however, this bioluminescence selection strategy may pick up many false positives due to the presence of the unintegrated, episomal reporter gene. The proportion of "germinal transgenic" embryos to "transfectgenic" embryos by the bioluminescence selection method remains unknown, but the results, obtained with PCR-based selection [10] suggest that bioluminescent embryos may still contain a dominant proportion of embryos with the unintegrated luciferase gene. Nevertheless, this approach contributes to a substantial reduction in the number of recipient cattle, to approximately one-fifth, and therefore is worth attempting.

Protein-based screening methods other than bioluminescence detection include the fluorescence analysis of bacterial $\beta$-galactosidase [66], and green fluorescent protein [52, 95], but in the fluorescence analyses by $\beta$-galactosidase and green fluorescent protein which are generally less sensitive than luciferase analysis, irradiation at about 480 and 360 nm excitation wavelength respectively, might be deleterious to embryo development.

Admittedly currently available DNA and protein-based selection methods do not make it possible to identify the integration status of transgenes in transplantable bovine oocytes; in principle, they merely suggest the presence of transgenes and transgene products. Although PCR analysis indicated 21% of the blastocytes carrying a transgene, only 7% of those PCR positive embryos were confirmed as truly transgenic [10], so that a further refinement of the currently available methods should be done or a totally different approach, not relying on PCR or on protein analyses, should be developed to increase the accuracy of transgene screening in live bovine embryos.

*(3) Gene constructs for improved gene expression*

For the production of high performance transgenic cattle, the way in which transgene expression is enhanced should be devised. The best and most expectable approach to this would be the use of knock-out and knock-in, i.e., endogenous-gene-replaced, cattle in which the lactoglobulin gene, for example, is substituted for a transgene encoding a pharmaceutical protein, but this will take a long time to accomplish, as there are at present no good ES and PG cell lines available for cattle. Although the nuclear transfer in combination with differentiated somatic cells has opened up a new path [131], the easiest approach to this subject would still

modify and optimize DNA constructs encoding pharmaceutical proteins in their structures.

In insertional mutations created by most of the gene transfer methods including microinjection and viral mediation, the extent of gene expression and the number of integrated copies of transgenes are not always correlated to each other. But the inclusion of certain DNA sequences such as a scaffold or matrix attachment region [7, 37, 54, 58, 115, 128], and a dominant or locus control region [24, 40, 118] allows tissue-specific and, to some extent, copy number-dependent and position-independent expression of transgenes. These regions had no effect on the gene activity in transient expression assays, whereas the effect was demonstrated in stably transformed animal cell lines and transgenic mice [99, 115]. Possible mechanisms of these specific DNA regions would be that they serve as the basis of chromatin loops, dividing the genome into separate structural units with an average size of 86 kb [53], and thereby might be able to insulate a transgene from expression-influencing effects exerted by the neighbouring host DNA as implicated by Breyne *et al.* [12].

Such insulating effects of neighbouring DNA could also be attained by introducing a fairly long DNA into which a transgene is inserted. Yeast artificial chromosomes (YAC) which have been exclusively utilized for genomic cloning [108] offer a possibility as they extend as long as several hundred kb or more. Indeed, Fujiwara *et al.* [34] reported that transgenic rats carrying only one copy of a 210-kb YAC DNA showed no position effect and high levels of human α-lactoalbumin exclusively in the mammary gland.

In addition to the inclusion of the aforementioned special DNA regions, silencer elimination [41] and intron inclusion [93] are also important factors to be considered. Particularly the latter finding suggests that the transgene should be derived from a genomic origin rather than cDNAs that are without introns. Together with the concern that transgene expression should be confined to the mammary gland for the secretion in the milk, promoters to confer transgene expression may well be limited to those switching genes encoding milk component proteins, i.e. α-lactoalbumin [45], β-lactoglobulin [112], β-casein [27], whey acidic protein [6], and α-$S_1$ casein [87]. By comparing the promoter strength and mammary gland specificity, Ninomiya *et al.* [87] concluded that in transgenic rats, the α-$S_1$ casein gene promoter was the most suitable for encompassing pharmaceutical proteins in rat milk. It is premature to state that this is true also in cattle.

Instead of modifying the target DNA structure itself, co-microinjection with another transgene might be useful in rescuing poorly-expressed transgenes. Improved gene expression in transgenic mice was demonstrated by co-integration of another transgene known to be efficiently expressed [22, 71]. The mechanism for this gene expression rescue is not yet clear. At least this transgene rescue in the mammary gland was found to be associated with transcription of the co-injected gene [133].

## Transfectgenic and Somatotransgenic Animals and their Applications

*(1) Localized in vivo gene transfer techniques*

In order to produce transfectgenic and somatotransgenic animals, foreign genes should be transferred somehow in tissues *in vivo*. Such gene transfer does not have to be systemic, but it should be sufficient to cover a local and limited area as the target. The most promising localized *in vivo* gene transfer (LIVGET) technique for producing somatotransgenic animals is the use of biological means, i.e. virus vectors. As Mulligan [74], and Mitani and Caskey [73] pointed out, however, the use of retrovirus vectors has often been restricted because of (1) the dependence of retrovirus entry into cells on the existence of the appropriate viral receptor in the target cells, (2) the necessity of cell replication for transgene integration, (3) the relatively labile property of retroviral particles in comparison to other viruses and low titers of up to $10^6$ infectious particles per ml, (4) the difficulty in keeping the safety standard from possible biohazard of virus replication and infection, and (5) more seriously the short maximum DNA fragment length to be inserted into the vector, up to 7 kb for retrovirus vectors [26, 31]. The last constraint severely limits the wide application of the retrovirus vectors as a means of DNA transfer.

Chemical or physical LIVGET techniques, on the other hand, require no such limitations with less chances of creating biohazard. Among the nonviral LIVGET methods reported in the literature, *in vivo* lipofection [90] or *in vivo* EP [75,76,81] may represent the most convenient and efficient means. Although the *in vivo* gene gun is another physical means possibly applicable to the bovine mammary gland, the DNA could reach only 2–3 mm deep from the tissue surface [129], which might result in poor gene expression, therefore being incapable of product evaluation. As stated earlier, integration of transfected genes cannot be expected by these nonviral LIVGET means so that animals thus produced become transfectgenic unless attempts are made to in-

crease integration frequency such as co-transfection of an integrase gene.

*(2) Comparison of nonviral gene transfer methods*

Direct comparison of nonviral gene transfer methods under *in vivo* conditions has scarcely been documented. Undoubtedly such comparison is quite difficult as each method has its own characteristics. The gene gun, for instance, is suitable for relatively thin and easily accessible tissues such as skin. Lipofection or lipoplex transfection is more suitable to larger areas of tissues or body for a transfection target than to localized areas because it can easily be completed by injecting lipid-DNA complex into the blood, although lipofection can also be applied directly in limited areas of target tissues. Direct injection of DNA is effectively done only in muscle and possibly in skin, but not in other tissues.

Nevertheless, the desire to answer the question that researchers often wish to know is overpowering: which nonviral method is the best one to choose? This section focuses on the comparison of nonviral methods under limited conditions. First, the target site is a limited area, equivalent to or less than 10 mm long × 10 mm wide. Secondly, gene expression is detected shortly after gene transfection such as within 24 or 48 h. This is necessary because gene expression will be greatly reduced as time goes by, which interferes with reliable comparison. Thirdly, the maximal amount of genes transfected is limited to 10 $\mu$g/site. In theory, unlimited amounts of DNA could be used by both EP and direct injection methods whereas there is a definite upper limit to the transfectable amount of DNA at one time for lipofection and gene gun methods since an optimal ratio of lipids or microparticles to DNA exists. Finally, target sites are limited to easily accessible tissues such as the mouse testis and the chicken embryo. For direct gene injection, muscle and possibly skin are the only tissues available, so that this method will be compared separately.

In the mouse testis, three nonviral gene transfer methods were compared, and it was found that *in vivo* gene gun and *in vivo* EP had higher CAT values than did *in vivo* lipofection or three *in vitro* methods, suggesting that EP and gene gun are better than lipofection under *in vivo* conditions [75]. In chicken embryos, a similar comparison was made between lipofection, gene gun and EP by using the lacZ reporter gene [76]. Typical examples of gene expression conferred by these three nonviral methods clearly indicated that EP was more efficient than the other two nonviral methods. Thus, in these tissues EP provides equivalent or even better transfer efficiency of foreign genes than lipofection or gene gun.

In the muscle, direct injection of various genes has been increasingly employed, especially for immunization [30, 109], although the mechanism of DNA uptake into muscle cells is unknown. Light pressure at the injection site or slight tissue damage is the most likely cause. In our preliminary experiment with rat abdominal muscles and mouse skin, direct injection of naked DNA gave detectable levels of gene expression, but *in vivo* EP showed more efficient foreign gene expression, approximately 15 to 50 times, than did direct DNA injection according to firefly luciferase activities [unpublished results].

In summary, although so far extensive comparison of gene transfer efficiency has not been done among nonviral methods, the data we obtained suggest that as far as a localized target area is concerned, *in vivo* EP would be one of the best choices as a nonviral gene transfer means.

*(3) Regulation of gene expression in transfectgenic animals*

Besides the transfer efficiency of foreign genes, a serious concern is whether or not the expression of transferred genes is properly regulated *in vivo* as was expected. A variety of questions may be raised as to the behaviour of transferred genes such as: how long does gene expression last?; is the tissue- or cell-specific gene expression maintained?, and, is the gene expression induced as intended? It is not yet possible to give satisfactory answers to all these questions. Instead, a few examples are presented, and adequate evaluation is left to those who wish to conduct detailed examination of controllable gene expression under any given condition. Unless otherwise indicated, the following findings in transfectgenic animals were derived by *in vivo* gene EP.

Duration of gene expression depends on the target tissue and probably to a certain extent on the plasmid construct. In the rat liver, lacZ reporter gene expression was maintained for at least 21 days after transfection, although the expression intensity decreased [44]. In our preliminary experiment with mice, luciferase gene expression in the liver lasted for only 7 days. Difference in the electrodes used might account partly, if not entirely, for the difference in the duration of gene expression: the former group of scientists used a hexagonal needle-array electrode, whereas in our study a pincette type electrode was used.

If muscles are the target site, the duration of gene

expression would be longer than that in any other tissues. In the rat abdominal muscle, firefly luciferase expression was maintained for at least 1 month [unpublished results]. In fact, in this tissue gene expression after simple DNA injection was maintained for several months [120, 121]. Therefore, for gene therapy where long-term gene expression is desired, application of *in vivo* EP to muscles would offer a good chance of supplying therapeutic and physiologically active proteins including hormones that are synthesized and secreted in the blood circulation.

In the testis, the duration of gene expression may be longer than that in the liver, but shorter than that in muscle. LacZ gene expression in the mouse testis was observed at two months after transfection [unpublished results]. Moreover, by using the *in vivo* gene gun method which would usually give weaker gene expression than *in vivo* EP, prolonged gene expression has been observed in the mouse testis at 1 month after gene transfection by including self-replicating sequences of Epstein-Barr virus [82].

Because both viral and nonviral LIVGET are generally conducted in a limited area of a target tissue, the expression of transferred genes is confined therein unless expressed proteins are secreted into the blood circulation. In this sense, gene expression is basically target area-specific with episomally present foreign genes even if a universal promoter is used, but under certain circumstances one might wish to express foreign genes only in a particular type of cell in a limited area of a target tissue. How faithfully is a foreign gene expressed in specific cells after being transferred? Although it is premature to conclude whether or not regulation of cell-specific gene expression is satisfactorily attained, our results imply that the exertion of cell-specific expression may be possible if a proper promoter is used to drive the transcription of a gene of interest. Substantially stronger CAT gene expression was found in the testis than in the leg muscle and liver of mice by using mouse protamin-1 promoter [80], which was deemed to act only in spermatids as demonstrated in the genuine transgenic study [134].

Over-expression of a foreign gene is not always sufficient for production and gene therapy, but gene expression should be induced or diminished as intended. Keys to solving this problem may lie in promoter constructs. For the inducible gene expression, a combination of the chicken oviduct, and promoters containing steroid response elements, i.e. MMTV-LTR and the chicken ovalbumin 5'-flanking sequences were used, since it is well known that gene transcription driven by these promoters is induced by steroid hormones [79, 106, 122]. As was expected, the results indicated that steroid induced CAT gene expression was conferred when driven by the MMTV-LTR and ovalbumin-900 promoters in the oviduct of living chickens, whereas the SV40 and ovalbumin-100 promoters that are without steroid response elements were uninducible by steroid hormones. These findings clearly support the hypothesis that controlled gene expression is possible in transfectgenic animals.

In practice, pharmacological doses of steroid hormone are not desirable. Instead, a tetracycline-dependent gene expression system could be used for both induction and repression of gene expression [49]. Alternatively, increasing attention may be paid to nutritional regulation as a milder and more preferable induction system. This possibility was demonstrated as shown in genuine transgenic mice in the literature [38, 70], fasting conferred a substantial increase likewise in reporter gene expression in the liver of transfectgenic mice when driven by the gene promoter encoding phosphoenol pyruvate carboxykinase, a key enzyme of gluconeogenesis, but not by the SV40 promoter [unpublished results].

*(4) Applications to production and therapeutics*

Application of transfectgenic and somatotransgenic animals for therapeutics and production has scarcely been reported. In general the level of foreign gene expression in these types of transgenic animals is weaker than that in genuine transgenic animals, but the most important characteristic of transfectgenic and somatotransgenic animals produced by LIVGET would be prompt gene expression after gene transfer, being as soon as several hours even in the mammary gland. In contrast, in genuine transgenic animals expressed proteins cannot be recovered from the mammary gland until adulthood. In cattle, for instance, it takes approximately two years from the genetic manipulation of one-cell embryos till the cows lactate. Therefore, under circumstances where quick production of pharmaceutical proteins is to be aimed at, transfectgenic and somatotransgenic animals would be of great value in the future.

On the use of somatotransgenic animals for pharmaceutical protein production, little information is available except for one example. By using a retrovirus vector, Archer *et al.* [3] demonstrated the introduction of the human growth hormone (GH) gene in the goat mammary gland, and its successful secretion in the milk from the following day, but the concentration of human GH

secreted into the goat milk was low, so that practical use must await further improvement of production efficiency.

For gene therapy in humans, somatotransgenic techniques have been discussed in detail in recent reviews [25, 125]. Therefore in the present paper, the description is restricted to some examples of the use of transfectgenic animals for therapeutic and production purposes.

Limited information is available on transfectgenic animals and humans, although some methods, especially *in vivo* EP, are efficient and widely applicable to various tissues. Perhaps the most successful applications of *in vivo* EP to gene therapy come from the studies with skin. Originally, *in vivo* EP was applied to this tissue to deliver drugs transdermally, and antibodies locally to various types of cancers [5, 68, 72, 91, 101]. Recently, however, depth-targeted gene delivery into skin was demonstrated in mice [135]. This would make it possible to treat skin cancers in combination with the use of tumor-suppressor genes such as p53 [9, 42, 86]. Brain cancer treatment is another example. In the rat brain tumor, local expression of the human monocyte chemoattractant protein-1 cDNA was transferred by *in vivo* EP, and expressed locally [89]. The presence of large numbers of macrophages and lymphocytes observed in treated tumor tissues indicates the potential of *in vivo* EP for gene therapy of brain cancer.

Phenotype manipulation in transfectgenic animals may offer an important application in the future. If *in vivo* EP is conducted in muscle with genes encoding therapeutic proteins to be secreted in the blood circulation, for example, long-lasting gene expression might be expected at a whole-body level. It was found in our preliminary experiments that growth of the chicken defective in the growth hormone receptor gene was significantly improved with the chicken IGF-I gene, and that plasma IGF-I was increased [unpublished results]. Calculation indicates that if similar growth promotion is to be attained exclusively by IGF-I protein infusion, the cost would be enormous, being as high as two to three thousand times that of *in vivo* gene EP treatment. Similarly, rat gastrin levels in plasma were significantly enhanced by *in vivo* EP with the rat gastrin cDNA for over 1 month [unpublished results]. The *in vivo* introduction of erythropoietin gene resulted in an increase in the packed cell volume from approximately 45% to over 70% maintained for several months [121, and unpublished results]. Such systemic treatments in transfectgenic animals and humans would further expand the possibility of therapeutics and production manipulation.

## Conclusion

The potential of genetic manipulation in animals now appears to be widely opened up by the recent development of animal cloning technology that surprisingly allows one to create a whole animal from the nucleus of cultured and differentiated cells. This breakthrough necessitates reappraisal and new classification of transgenic animals as a classical scientific term. In the present review, different transgenic names, i.e. genuine transgenic, germinal transgenic, somatotransgenic and transfectgenic, and their definitions have been proposed. The key step common to these novel cells and animals is gene transfer. With this process in mind, emphasis has been placed upon how these transgenic cells and animals are related to each other, and how they are produced and applied for production and therapeutic purposes. At present each gene transfer technique suffers from specific drawbacks that are inherently involved in their own systems, but there is no doubt that the shortest way to approach their practical applications is in the effort to improve and attain gene transfer efficiency which becomes sufficient for meeting imposed prerequisites for particular aims and goals.

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