Transgenic Animals

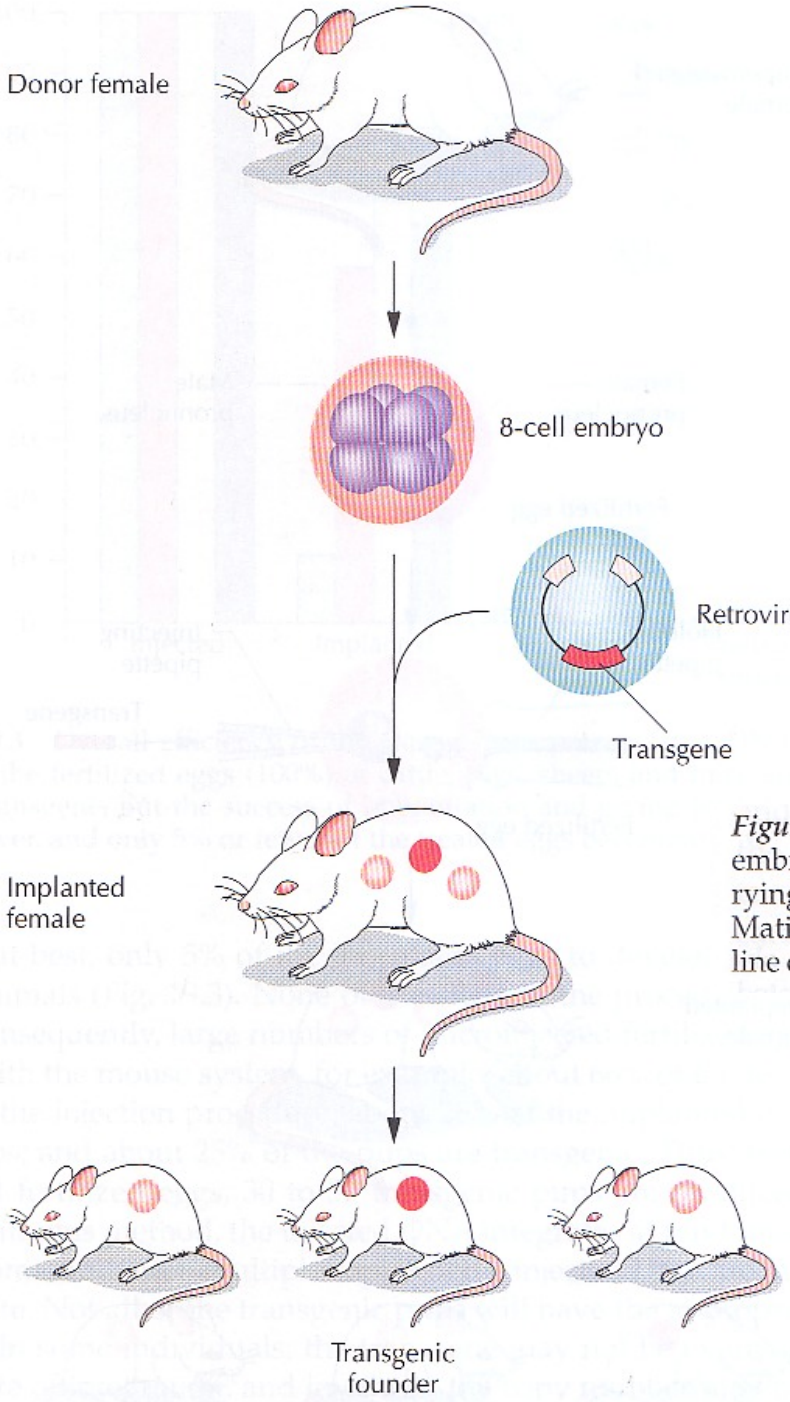

Figure 19.1 Establishing transgenic mice with retroviral vectors. Cleavage-stage embryos, usually at the eight-cell stage, are infected with a defective retrovirus carrying a transgene. Implanted females (foster mothers) give birth to transgenic pups. Matings are carried out to determine which pups have the transgene in their germ line cells. Transgenic lines can be established from these founder transgenic animals.

Transgenic Animals

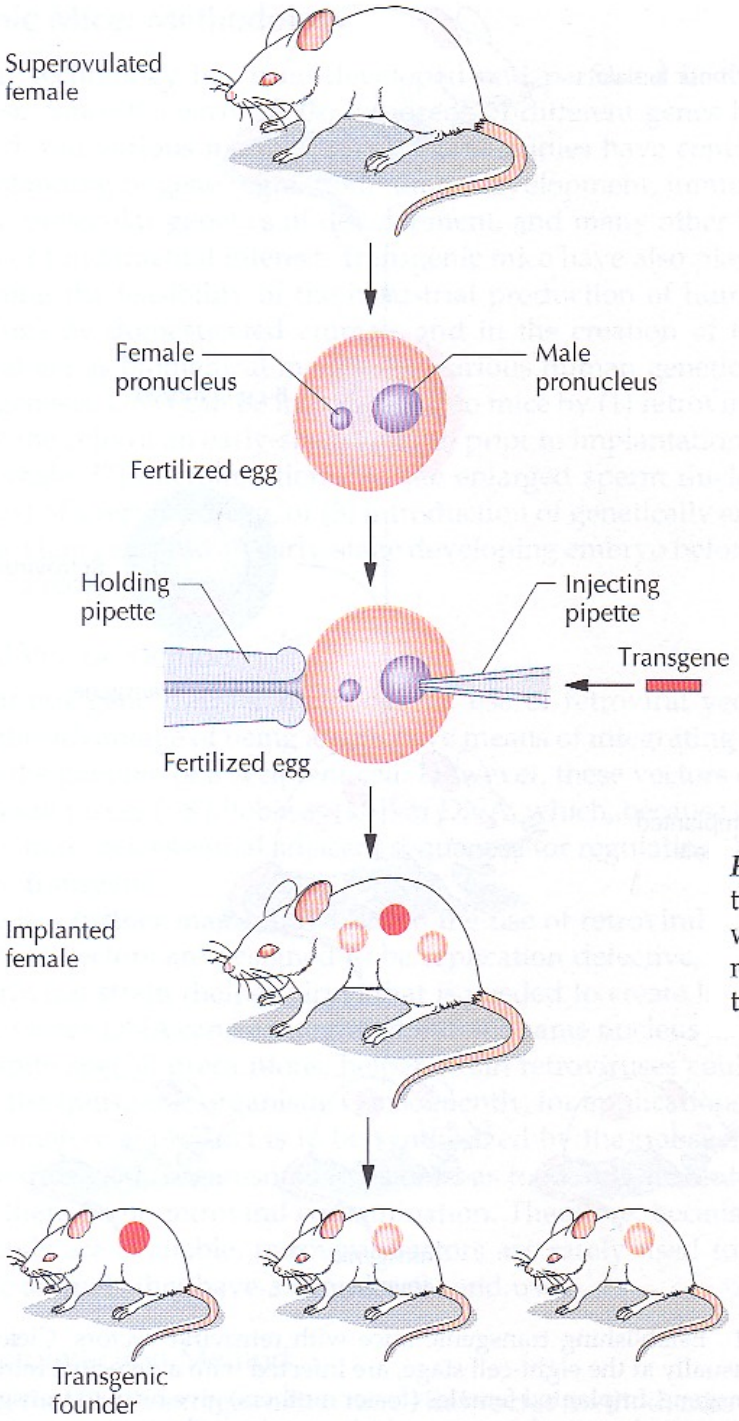

Figure 19.2 Establishing transgenic mice by DNA microinjection. Eggs are obtained from donor females that have been induced to superovulate and then mated with males. Purified samples of the transgene construct are microinjected into the male pronucleus of a fertilized egg. Implanted females (foster mothers) give birth to transgenic pups from which transgenic lines can be established.

Transgenic Animals

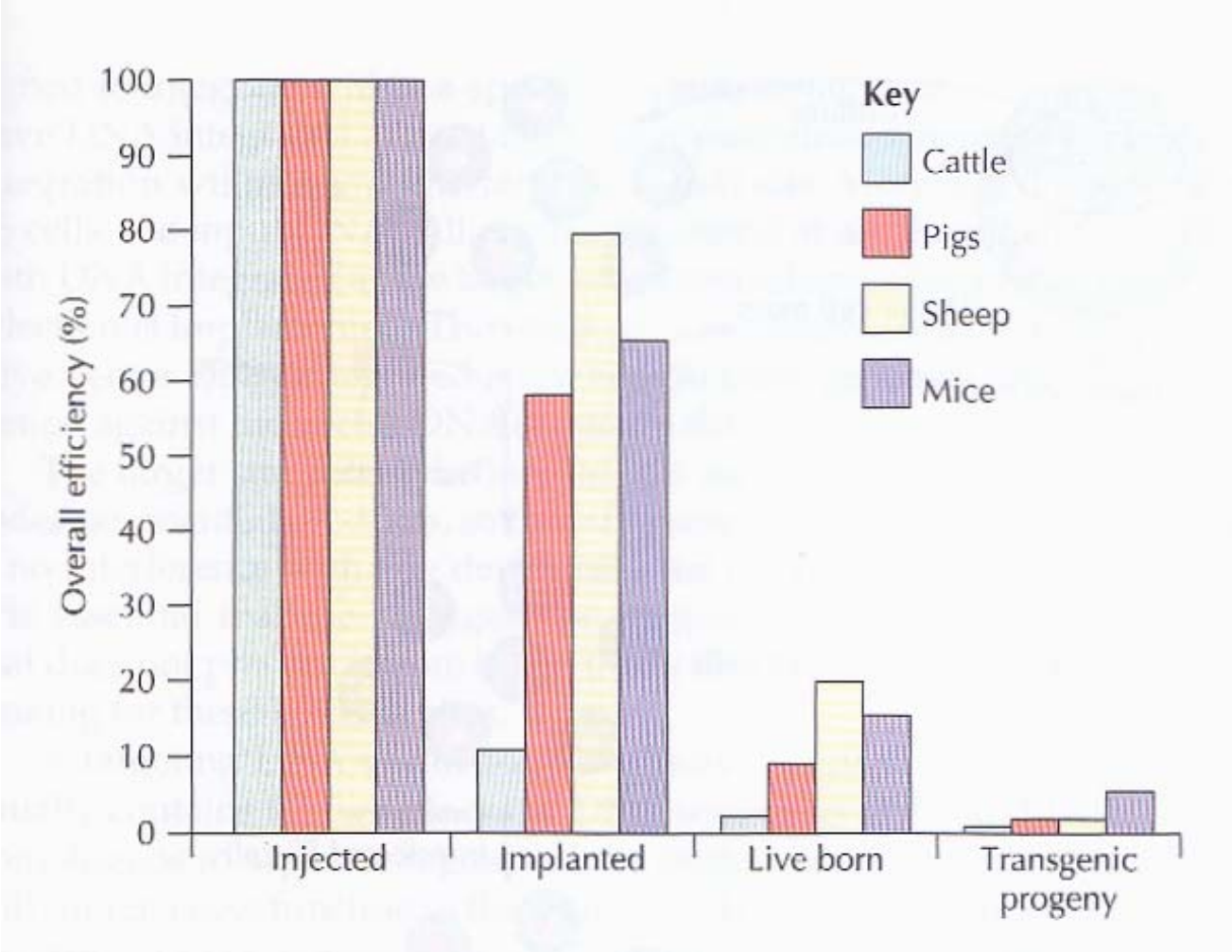

Figure 19.3 Overall efficiency of the transgenesis process after DNA microinjection. All the fertilized eggs (100%) of cattle, pigs, sheep, and mice are inoculated with a transgene, but the success of implantation and giving birth to offspring is much lower, and only 5% or fewer of the treated eggs become transgenic progeny.

Transgenic Animals

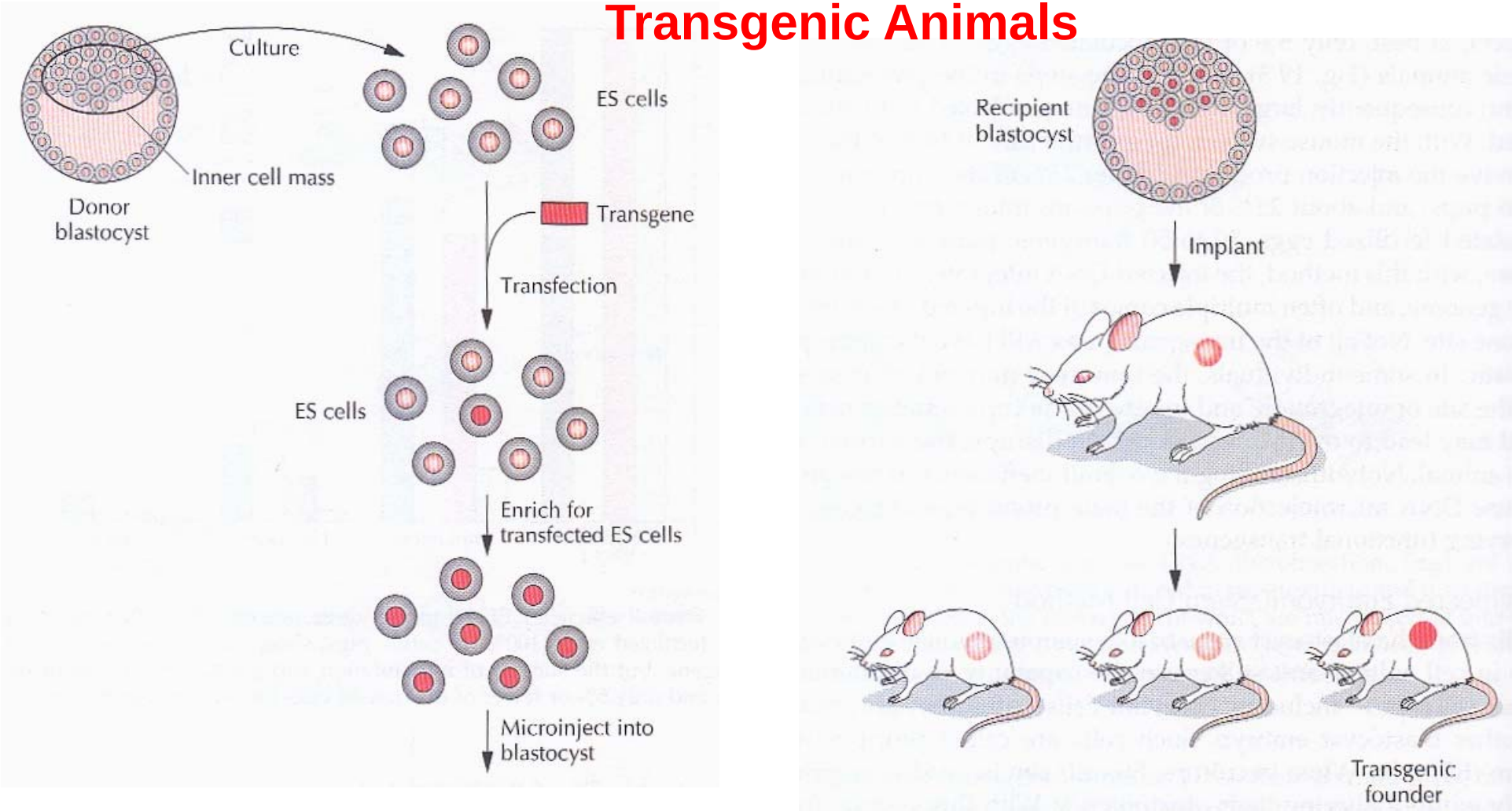

Figure 19.4 Establishing transgenic mice with genetically engineered ES cells. An ES cell culture is initiated from the inner cell mass of a mouse blastocyst. The ES cells are transfected with a transgene. After growth, the transfected cells are identified by either the positive-negative selection procedure or PCR analysis. Populations of transfected cells can be cultured and inserted into blastocysts, which are then implanted into foster mothers. Transgenic lines can be established by crosses from founder mice that carry the transgene in their germ lines.

Transgenic Animals

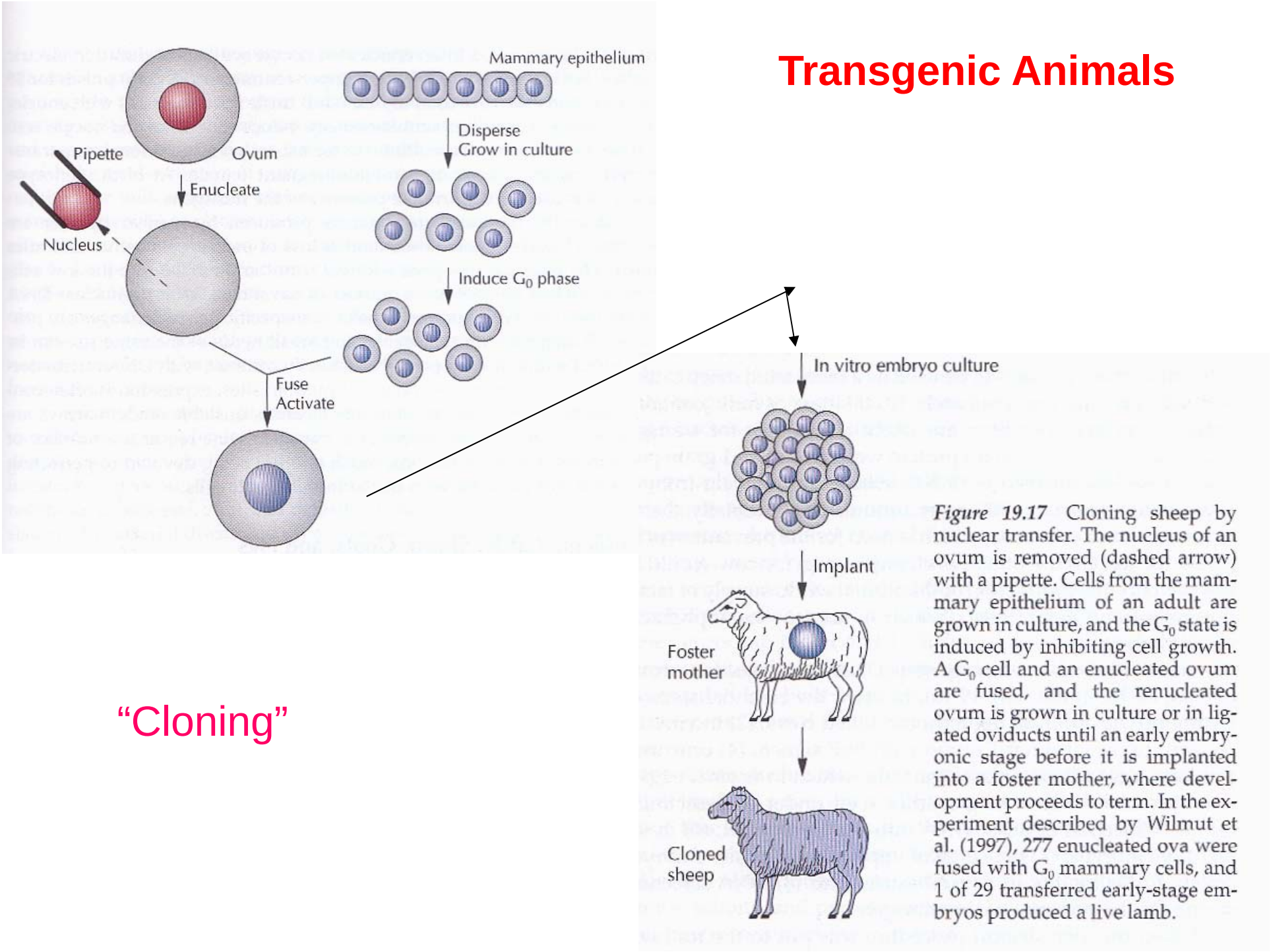

Figure 19.17 Cloning sheep by nuclear transfer. The nucleus of an ovum is removed (dashed arrow) with a pipette. Cells from the mammary epithelium of an adult are grown in culture, and the G_0 state is induced by inhibiting cell growth. A G_0 cell and an enucleated ovum are fused, and the renucleated ovum is grown in culture or in ligated oviducts until an early embryonic stage before it is implanted into a foster mother, where development proceeds to term. In the experiment described by Wilmut et al. (1997), 277 enucleated ova were fused with G_0 mammary cells, and 1 of 29 transferred early-stage embryos produced a live lamb.

"Cloning"

Table 19.3 Some exogenous proteins that have been expressed in the mammary glands of transgenic animals

Antithrombin III
Calcitonin
Erythropoietin
Factor IX
Factor VIII
Fibrinogen
Glucagon-like peptide
Granulocyte colony-stimulating factor
Growth hormone
Hemoglobin
Human serum albumin
Insulin
Insulin-like growth factor 1
Interleukin 2
Lactoferrin
Lysozyme
Monclonal antibodies
Nerve growth factor β
Protein C
Superoxide dismutase
Tissue plasminogen activator
α1-Antitrypsin
α-Glucosidase
α-Lactalbumin

Transgenic Animals

Transgenic Animals

Tissue specific Expression

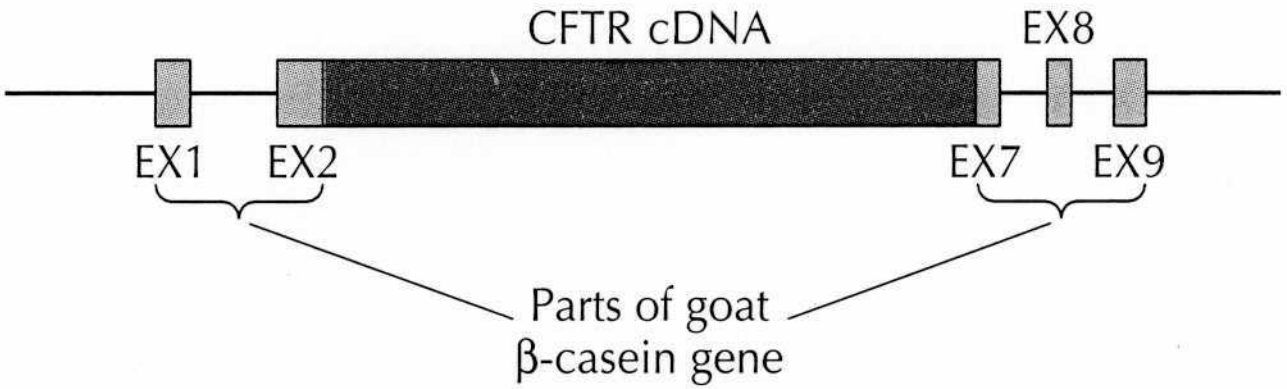

Figure 19.12 Goat β-casein gene–CFTR cDNA expression construct. The full-length cDNA for CFTR was cloned between exon 2 (EX2) and exon 7 (EX7) of the goat β-casein gene. The promoter and transcription termination sequences and exons 1, 8, and 9 (EX1, EX8, and EX9) of the casein gene are retained.

CFTR: Cystic Fibrosis Transmembrane Conductance Regulator

Transgenic Animals

Tissue specific Expression

Table 19.2 Mammary gland transgenes, promoter sequences, and recipient organisms

Transgene	Promoter	Transgenic species
Longer-acting tissue plasminogen activator	Whey acidic protein	Goat
α_1-Antitrypsin	β-Lactoglobulin	Sheep
Clotting factor IX	β-Lactoglobulin	Sheep
Soluble CD4 protein	Whey acidic protein	Mouse
Lactoferrin	α_{s1}-Casein	Cattle
Urokinase	α_{s1}-Casein	Mouse
CFTR	β-Casein	Mouse
Interleukin-2	β-Casein	Rabbit

Transgenic animals as organ supplier

Humanizing the pig:

Introduction of Human Genes

Knock out of pig genes → α-1.3 galactosyltransferase → avoid immunogenic reaction

Immunological Barriers

Hyperacute rejection
Acute vascular rejection
Cellular rejection
Chronic rejection

Porcine endogenous retroviruses

Biopharmaceutical production in transgenic livestock

Natalie S. Rudolph

The production of recombinant human proteins in the milk of transgenic dairy animals offers a safe, renewable source of commercially important proteins that cannot be produced as efficiently in adequate quantities by other methods. A decade of success in expressing a variety of proteins in livestock has brought three human recombinant proteins to human clinical trials. Recent progress has drawn on molecular biology and reproductive physiology to improve the efficiency of producing and reproducing useful transgenic founder animals, and to improve the expression of heterologous proteins in their milk.

Within six years of the approval of the first recombinant therapeutic product [human insulin, in 1982 (Ref. 1; http://www.phrma.org/charts/biochart.html)], the first recombinant human protein was expressed in the milk of a transgenic dairy animal (Factor IX in sheep[2]). In the decade since then, milk expression has been reported for at least 17 different proteins in five livestock species, 11 of them at commercially feasible levels of ≥ 1 g l^{-1} (Table 1). Concomitant advances have been made in purifying proteins from raw milk[3,4]. By 1998, three transgenically produced recombinant proteins were in clinical trials: anti-thrombin-III from transgenic goats (Phase III), α1-antitrypsin (α1-proteinase inhibitor) from sheep (Phase II), and α-glucosidase from transgenic rabbits (Phase I, now complete)[5].

Expression of recombinant human proteins in the milk of transgenic dairy animals offers a safe and renewable source of clinically important proteins that cannot be produced as efficiently in adequate quantities by other methods. Other recent reviews have summarized transgenic methods[6–8], protein purification[3], post-translational processing[9,10], economics[11], commercial development[5,12] and safety and regulatory issues[3,13]. Technical overviews of selected topics in large-animal transgenics and related topics are available at a number of web sites (Box 1). This article focuses on recent progress in increasing the efficiency of producing and reproducing transgenic founder animals.

Transgenic methods

The basic strategy for producing transgenic animals has not changed much over the past decade. A gene construct combining the target heterologous gene linked to a promoter from a milk-specific protein is introduced into fertilized embryos, most commonly by microinjection through a fine glass needle. Embryos are cultured briefly and then transferred to hormonally synchronized females for gestation[14]. First-generation transgenic animals ('founders') are identified by postnatal biopsy and are bred at maturity to confirm transgene transmission.

If the founder is female, then milk is available for recombinant-protein analysis when she delivers her own young; however, a male founder must be bred to produce daughters, which will lactate when they are bred. Hormonal induction of lactation in prepubertal goats, cows and pigs – both female and male – can speed the recovery of sufficient quantities of milk to assess expression levels, to begin protein characterization and sometimes even to start protein-purification-process development.

Heterologous proteins have been expressed in the obvious dairy species: cows, sheep and goats. However, pigs and rabbits are also used for selected applications because of their large litters and shorter generation times (Table 2). The size of the production herd required for a particular application will depend primarily upon total annual need, the recombinant-protein expression level and the recovery efficiency (Box 2).

Transgene integration into the host chromosome is a rare and random event, making founder production time consuming and inefficient (Table 3). In a new gene-transfer method described recently, a replication-defective retroviral vector carrying a transgene construct was microinjected into unfertilized bovine oocytes[15]. After *in vitro* fertilization, the transfer of ten embryos into gestation recipients produced four live-born calves, all of which were transgenic. These calves carried a single-copy insertion, which was transmitted from one bull in approximately mendelian ratios[15]. The broad host range of retroviral vectors makes them potentially useful for other livestock species as well but they have several drawbacks, including limited insert size, potential interference of retroviral sequences with transgene regulatory elements, and biosafety and regulatory issues.

Recent transgenic successes

Strategies have been developed to express a variety of interesting recombinant proteins – large and small, simple and complex – with high efficiency and full bioactivity. The largest and most complex protein successfully produced to date is the human clotting protein Factor VIII, a large heterodimer that was correctly processed to a bioactive protein in pigs[16]. The gene contains 26 exons and the cDNA alone is 7.6 kb long.

At the other extreme, small peptides are unstable in biological systems, but they can be expressed in transgenic animals if fused to a carrier protein. For example,

N. S. Rudolph (nrudolph@ix.netcom.com) is at 144 Mile Hill Road, Boylston, MA 01505, USA.

0167-7799/99/$ – see front matter PII: S0167-7799(99)01341-4

Table 1. Human biopharmaceuticals produced in the milk of transgenic farm animals

Expressed protein	Transgene source	Promoter	Expression level (mg ml^{-1})	Ref.
Cow				
Lactoferrin	cDNA	Bovine α-s1 casein	ND	70
Human α-lactalbumin	NA	NA	2.4	17
Goat				
Anti-thrombin-III	NA	Caprine β-casein	14	3
α1-Antitrypsin (α1-proteinase inhibitor)	NA	Caprine β-casein	20	3
Growth hormone	NA	Retrovirus	1.2×10^{-4}	71
Monoclonal antibody (colon cancer)	Genomic	Caprine β-casein	10	3
Tissue-plasminogen activator, long-acting variant	cDNA	Caprine β-casein	6	27
Pig				
Factor VIII	cDNA	Murine WAP	3	16
Protein C	cDNA	Murine WAP	1	72
Rabbit				
Calcitonin	Fusion protein	Ovine β-lactoglobulin	2.1	17
Extracellular superoxide dismutase	cDNA	Murine WAP	2.9	19
Erythropoietin	cDNA	Rabbit WAP protein	0.05	73
Erythropoietin	Fusion-protein cDNA	Bovine β-lactoglobulin	0.05	30
Growth hormone	Genomic	Murine WAP	0.05	74
Insulin-like growth factor 1	cDNA	Bovine α-s1 casein	1	75
Interleukin-2	Genomic	Rabbit β-casein	0.0005	76
Sheep				
α1-Antitrypsin	Minigene	Ovine β-lactoglobulin	35	31
Factor VIII	cDNA	Ovine β-lactoglobulin	ND	77
Factor IX	cDNA	Ovine β-lactoglobulin	0.005	78
Fibrinogen	Genomic	Ovine β-lactoglobulin	5	20

Abbreviations: NA, not available; ND, not determined; WAP, whey acidic protein.

the salmon calcitonin peptide was fused to a small milk protein (human α-lactalbumin) and successfully expressed and amidated in rabbits. Calcitonin was cleaved from the fusion precursor *in vitro* during the purification process to yield a peptide with potent bioactivity *in vivo*[17]. This is one approach to expressing physiologically active polypeptides without compromising the physiology or health of the transgenic production animal.

Proteins that require post-translational modification (e.g. glycosylation) have been expressed successfully, including anti-thrombin-III in goats[9] and Protein C in pigs[10]. Cystic-fibrosis transmembrane-conductance regulator (CFTR), an intrinsic membrane protein that is not ordinarily secreted, has been targeted to milk globules for expression in the milk of transgenic mice[18]. Extracellular superoxide dismutase, a complex *N*-glycosylated homotetramer that carries copper and zinc atoms and is sensitive to proteolysis, has been produced in physiologically active form in transgenic rabbits[19]. Even multimeric proteins have been expressed and assembled *in vivo* by the coinjection of separate transgene constructs containing the individual protein chains. Correct assembly into bioactive proteins requires coexpression of the individual proteins at the same time in the same cell with the correct stoichiometry. This has been accomplished *in vivo* for heterodimeric monoclonal antibodies expressed in mice and goats[3], and for heterotrimeric fibrinogen produced in sheep[20].

Current technical challenges

Despite these accomplishments, significant technical challenges remain for optimizing protein expression for commercial production. There is a need for increased product development efficiency, lower costs and, for potential biotherapeutics, a shorter time to clinical trials. Particular attention is being focused in two areas: maximizing heterologous-protein expression levels and maximizing the efficiency of producing founder animals.

Ideally, heterologous-protein expression levels should be related to the number of integrated transgene copies (copy-number dependence) and should not be limited by the location of that integration site within the host genome (position-independent expression). In practice, this is not always the case. Several approaches have been taken to overcome these problems, including use of isolated regulatory elements to increase expression, the use of special sequences to insulate transgenes from local chromosomal-position effects and the development of larger vectors to accommodate protein-coding sequences along with exogenous regulatory elements.

Promoters

Most of the regulatory elements that control the timing and location of gene expression reside in the promoter region within a few kilobases of the 5′ end of the structural gene. Heterologous gene expression is targeted specifically to the lactating mammary gland by use of promoters isolated from milk-specific genes[3,21–23]. Milk-protein promoters have been isolated and characterized from mice, rats, goats, guinea pigs, cows, rabbits and sheep[3]. Most of these promoters function across species barriers, including the promoter for murine whey acidic protein (WAP), a protein that occurs naturally in rodents but not in ruminants. However, the promoters used most commonly for commercial transgenic pharmaceutical production are ovine β-lactoglobulin (BLG), caprine β-casein and bovine α-s1 casein, which are used in their respective species, and WAP, which is used in pigs and rabbits.

Enhanced transgene expression

Isolated promoter sequences are not always sufficient for accurate control of expression level, timing and site specificity. Usually, transgenes are expressed more consistently and at higher levels from full genomic sequences than they are from cDNA sequences[24–26], although there are exceptions[16,27,28]. Functional dissection of these regulatory regions suggested the presence of enhancers within genomic introns, particularly in the first few introns at the 5′ end.

The problem is that many target proteins of interest are too long to be incorporated *in toto* into the commonly used phage and cosmid cloning vectors. This limits both the size of the genes that can be cloned out of the genome and the size of the fusion construct that can be used for transgenic production. Therefore, attempts have been made to minimize the construct size by using shorter cDNA sequences and adding defined regulatory elements to the constructs. For example, modifying the α1-antitrypsin cDNA to include the first few introns of the genomic sequence was effective for increasing the expression level of this protein in transgenic mice[29]. A similar approach also worked for BLG[26]. Erythropoietin was expressed from a bovine BLG promoter in rabbits after insertion of the protein cDNA sequences into the first genomic intron of BLG, used here as a promoter[30]. Alternatively, intronic regulatory elements may be drawn from the promoter rather than from the target gene. The insertion of a 7.2 kb Factor-VIII cDNA into the first intron of the genomic WAP sequence permitted Factor VIII expression in the milk of pigs[16].

Intrinsic regulatory elements have been found in distal introns of α1-antitrypsin[31] and in both proximal and distal introns of BLG[26]. Others have been found in the promoter region, such as the binding site for milk-protein-binding factor (MPBF; also known as mammary-gland-specific factor, MGF) located in the BLG

Box 1. Non-rodent transgenics and cloning websites with useful technical information and links. This list does not include sites dealing only with cloning ethics or only with transgenic mice

Transgenics

Animal-genetic-engineering news
http://www.biol.tsukuba.ac.jp/~macer/NBBDM.html
http://www.biol.tsukuba.ac.jp/~macer/NBBGEA.html
Extensive annotated chronological bibliographies of large-animal transgenics publications and some news.
http://www.biol.tsukuba.ac.jp/~macer/NBB.html
Bimonthly updates of the above two sites and other associated information.
From Eubios Ethics Institute, Christchurch, New Zealand.

ATP on-line workshop on transgenics and cloning
http://atp.nist.gov/atc/atc_off.htm
Technical summaries and responses by invited scientists, posted September–October 1998. From the Advanced Technology Program of the National Institute of Standards and Technology, USA.

Mammary-gland biology
http://mammary.nih.gov/
Mammary-gland Information Core (MAGIC). Full text minireviews of mammary genes and targeted genetic modification. From the Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, USA.

Mammary-transgene reviews
http://www.biomedcomp.com/
Annotated bibliography of selected reviews of mammary transgenesis through 1996. From Baylor College of Medicine and Biomedical Computing, Houston, TX, USA.

Roslin Institute transgenics and cloning site
http://rio10.ri.bbsrc.ac.uk/library/research/cloning/cloning.html
Press releases, illustrated technical notes and external links relating to genome mapping, cloning and nuclear transfer.
http://www.ri.bbsrc.ac.uk/bioinformatics/databases.html
Searchable database of pig, cow and sheep genes.
From Roslin Institute, Roslin, Midlothian, UK.

Mammalian cloning and nuclear transfer

BioSpace cloning site
http://www.biospace.com/whats_new/dolly.cfm
Links to general cloning information and news; updated frequently. From Synergistic Media Network, San Francisco, CA, USA.

Bovine cloning
http://www.infigen.com/home.htm
Unpublished technical information on successful bovine cloning reported in the press. From Infigen, a division of ABS Global, Deforest, WI, USA.

New Scientist 'Cloning Special Report'
http://www.newscientist.com/nsplus/insight/clone/clone.html
Selected recent articles; links to sites with technical and general information on large animal cloning. By *New Scientist* magazine.

PhRMA genomics and cloning site
http://www.phrma.org/genomics/cloning/index.html
Links to technical and general information on mammalian cloning. Some pages incomplete, some links outdated or directed to amateur sites. By Pharmaceutical Research and Manufacturers of America, Washington, DC, USA.

Scientific American Explorations expanded cloning features
http://www.sciam.com/explorations/
Expanded website with technical information from *Scientific American* articles and external links.
By *Scientific American Explorations* magazine.

Yahoo 'Cloning Update' site
http://headlines.yahoo.com/Full_Coverage/Tech/Cloning/
Extensive site with links to recent articles and other web sites relating to cloning.

468

Table 2. Production characteristics of selected livestock species used as transgenic bioreactors

	Rabbit	Pig	Sheep	Goat	Cow
Gestation time, months	1	4	5	5	9
Age at sexual maturity, months	5	6	8	8	15
Time between transgene introduction and first lactation, months					
Founder females					
Induced lactation in prepubertal founder females	–	–	9	9	16
Natural lactation in founder females	7	16	18	18	33
Founder males					
Induced lactation in prepubertal daughters	–	–	22	22	45
Natural lactation in daughters	15	28	31	31	57
Number of offspring	8	10	1–2	1–2	1
Annual milk yield, l	4–5[a]	300[b]	500	800	8000
Raw recombinant protein per female per year, kg	0.02	1.5	2.5	4	40

[a]Average total production from 2–3 lactations per year.
[b]Average total production from 2 lactations per year.

promoter[23]. Specific regulatory elements now are being identified and isolated for use in transgene constructs.

Another strategy for increasing expression is transgene rescue, in which a poorly expressing transgene construct is coinjected with a construct known to be highly expressed. Coinjected constructs tend to integrate together at a single chromosomal site. Coinjected full-length BLG genes restored high-level expression of α1-antitrypsin and Factor-IX cDNA constructs in transgenic mice, increasing both the frequency and the maximal level of transgene expression[32]. However, transgenic rescue did not work when both genomic and cDNA sequences from the same murine WAP gene were injected into transgenic mice[33].

Position independence

Transgene integration into the host genome is random and the chromosomal integration site dramatically affects heterologous-protein expression levels. Some chromatin regions are more permissive for gene expression than others. This may be due to the presence of *cis*-acting repressors, the absence of required enhancers, the effect of chromatin configuration in regulating the accessibility of genes to transcription factors or proximity to heterochromatic regions of the chromosome.

Certain transgenes that do exhibit position-independent expression from genomic constructs, such as ovine BLG expressed in transgenic mice[32], have been instructive. Position independence of this construct was lost when selected combinations of introns were removed[34] and when the transgene was integrated into heterochromatic or repetitive chromatin[35]. In the latter case, two mouse lines in which the transgene had integrated near the centromere showed position-effect variegation: variable protein-expression levels between animals within a line, which corresponded to mosaic transgene expression in patches of epithelial cells in the mammary gland. A third line carrying the same construct as one of the variable lines that was integrated more distally on its chromosome showed consistent expression levels between individuals.

Box 2. Estimated transgenic herd size to produce representative recombinant proteins

The herd size required to meet total annual production needs may be estimated from the following equation.

$$\text{Herd size} = 1.3 \times \frac{\text{Total annual need (g)}}{\text{Expression level (g l}^{-1}\text{)} \times \text{Total milk volume (l)} \times \text{Purification efficiency (\%)}}$$

The factor of 1.3 accounts for the observation that, at any given time, 30% of the animals in a dairy herd are typically not lactating. The approximate numbers of transgenic females needed to produce several representative recombinant proteins have been estimated from this equation, assuming an average protein-expression level of 5 g (l milk)$^{-1}$ and a 60% protein-recovery efficiency (Table I).

Table I. Herd-size estimates for various species and products

Protein	Estimated need (kg y^{-1})	Species	Herd size
Human serum albumin	100 000	Cow	5400
α1-Antitrypsin	5000	Sheep	4300
Monoclonal antibody	100	Goat	58
Anti-thrombin-III	75	Goat	43
Factor IX	2	Pig	4

Table 3. Efficiency of producing transgenic animals[a]

	Sheep	Ref.	Cows	Ref.	Goats	Ref.
Reconstructed or microinjected oocytes developing to blastocysts						
Microinjection	61/507 (14%)	67	174/836 (21%)	15	770/1130 (68%)[b]	79
Nuclear transfer	47/120 (51%)	62	22/99 (22%)	47	89/220 (40%)	60
Embryo transfers resulting in transgenic offspring						
Historical literature before 1996[c]	1/380 (0.3%)		1/520 (0.2%)			
Microinjection					4/770 (0.5%)	79
retroviral vector			4/10 (40%)	15		
biopsied embryos			1/4 (25%)	51		
			7/21 (33%)	50		
Nuclear transfer	5/67 (7%)	67	6/10 (60%)	45	3/85 (4%)	60
Live births producing transgenic founders						
Historical average[c]	5–10%	3	5–10%	3	8–10%	3
Microinjection	56/1286 (4%)	67	4/4 (100%)	15	4/86 (5%)	79
Nuclear transfer	5/6 (83%)	67	6/7 (86%)	47	3/3 (100%)	60
Number of living animals required to produce one transgenic founder						
Microinjection	51	67	5[d]	15		
Nuclear transfer	21	67			43	60

[a]Published data are available only for cows and sheep, and limited data for goats, although success in other species has been reported anecdotally.
[b]Inferred from reported data.
[c]Average figures.
[d]Oocytes were collected postmortem, eliminating the need for live donors.

Genomic constructs of β-globin genes also show position-independent expression, which has been attributed to locus-control regions (LCRs). Isolated LCRs from the human β-globin gene cluster[36] and the human growth-hormone gene cluster[37] have been used as extrinsic *cis*-acting elements to confer position-independent expression on their respective single-copy transgenes in mice. Both of these LCRs are multicomponent complexes that contain four DNAse-I-hypersensitive sites and are located some distance upstream of their respective coding sequences. The β-globin LCR has been dissected and a minimal 6.5 kb microlocus containing a critical 200–300 b core has been isolated for use in transgenesis[36]. LCRs have not been found in milk-protein genes and so it will be important to confirm that these isolated elements will work on coding sequences from heterologous genes expressed in the mammary gland.

A second type of regulatory element that confers position independence is the matrix-attachment region (MAR), which serves as a boundary element between adjacent genetic domains. MARs may be isolated and used as 'insulators' to shield transgene expression from the effects of neighboring chromatin. A MAR isolated from the chicken lysozyme gene has been used to confer position-independent expression on mice carrying a murine WAP construct with a truncated promoter that was poorly expressed when used alone[38].

New vehicles for long constructs

Vector-size constraints can be circumvented with vectors that can accommodate regulatory elements along with large protein-coding sequences. Yeast artificial chromosomes (YACs), for example, can carry up to 2 Mb of insert DNA, compared with the 40–50 kb capacity of phage or cosmids[39]. YACs bearing the human β-globin cluster confirmed the role of LCRs in regulating these genes in transgenic mice[40]. Transgenic rats carrying the human α-lactalbumin gene on a 210 kb YAC efficiently secreted this protein into their milk at levels up to 5 mg ml^{-1}, with no apparent position effects[41]. Four of the ten transgenic founders expressed and transmitted the transgene, significantly more than the 5–10% typically observed with conventional transgene vectors[3]. Although transgenic rabbits[42,43] and pigs[44] have been produced with YACs, YAC-facilitated milk expression of heterologous protein in other livestock species has not been reported.

Even larger vectors are being developed from mammalian artificial chromosomes. Like YACs, these human and murine constructs are autonomously replicating and segregating episomes that may be transferred to recipient cells by cell fusion or viral encapsidation[45]. Murine satellite-DNA-based artificial chromosomes (SATACs) with a 4 Mb insert have been maintained under selection in culture for more than two years[46]. However, the production of transgenic animals from these vectors has not yet been reported.

Embryonic stem cells

Transgene microinjection permits only the random insertion of new heterologous genes into the host genome. Elegant, efficient and powerful methods of genetic manipulation, including targeted genetic modification by selective knock-out or replacement, are possible for cells that can be cultured and selected *in vitro*. Embryonic stem (ES) cells are pluripotent cell lines that can proliferate *in vitro* in an undifferentiated state, can be induced to undergo differentiation *in vitro* along several different developmental pathways and can contribute to the development of a chimeric animal when combined with an early host embryo. Murine ES cells have been used to generate a wide variety of

transgenic mouse lines. Despite years of effort, however, stable and reliable ES cell lines have not been developed for any other species.

Nevertheless, so-called 'ES-like' cells have been used to produce livestock. Cell lines derived from both fetal fibroblasts and blastocyst inner cell mass have been used to produce transgenic chimeric calves[47], and embryonic germ-cell lines established from fetal primordial germ cells have been used to produce transgenic piglets[48]. Germ-line transmission of transgenes from these animals has not yet been reported. This approach has some limitations: the method of mixing transgenic pluripotent cells with normal blastocysts creates chimeric animals that usually contain the transgene in some, but not all, tissues. A second generation is required to breed and identify animals that are uniformly transgenic – that is, that contain transgene in the mammary gland, to permit recombinant-protein expression in milk, and in germinal tissue, to permit transgene transmission to progeny.

Maximizing production efficiency

Two critical milestones in the transgenic process are: (1) the first collection of milk containing recombinant protein for initial characterization; and (2) the first production of bulk protein for clinical trials. Efforts to maximize the efficiency of producing and reproducing commercially important transgenic animals have focused on increasing the overall yield, reducing the number of non-transgenic animals that must be produced and tested, and decreasing the effective intergenerational time. Assisted-reproduction technologies such as superovulation, artificial insemination, *in vitro* embryo culture and manipulation, and embryo splitting and transfer are used extensively in the dairy and beef industries, where they are the primary means for reproducing elite genetic stocks. Adopting these methods for transgenic-animal production could reduce the number of animals required, lower founder-production costs and potentially avoid the seasonal-breeding issues reported for sheep and goats.

Transgenic animals generally carry the recombinant construct at a single chromosome insertion site and hence they are hemizygous for the transgene. Only half of their offspring, on average, will inherit this transgene. This 50% inefficiency increases the number of animals needed to expand a transgenic-production herd, doubling the required number of oocyte donors and embryo recipients, as well as the number of pregnancies to maintain and offspring to analyse for the presence of transgene.

However, '*in vitro* progeny testing' can shorten the time required to identify founders and theoretically increase the production efficiency to 100%. Preimplantation genetic analysis of biopsied embryos can identify their sex and detect the transgene before implantation, so that only transgenic embryos of the desired sex are transferred into recipients and carried to term. Specific genes and chromosomes may be detected by fluorescent *in situ* hybridization (FISH) or PCR by methods that are sensitive enough for preimplantation genetic analysis on a single embryo cell[49]. Preimplantation analysis of bisected embryos has been used successfully to produce selected transgenic calves[50,51]. In the latter example, bacterium-specific N^6-adenine-methylated DNA was introduced into the construct during plasmid propagation. A PCR assay was then devised that used selective cleavage of the modified site by the *DpnI* restriction endonuclease, together with the *Bal31* exonuclease, to distinguish integrated transgenes from unincorporated plasmids that persist in morulae and blastocysts. Simultaneous detection of transgene and Y-chromosome sequences was performed in less than 6 h.

Prepubertal reproduction is now possible in cows. Mammalian oocyte production begins early in fetal development and ends around birth, and neonatal ovaries contain up to 300 000 oocytes[52]. Oocytes can be collected from superovulated heifers as young as 2–4 months, followed by *in vitro* fertilization and transfer to surrogate mothers[53]. Minimally invasive methods for ultrasound-guided transvaginal oocyte retrieval (TVOR; also called oocyte pick-up, OPU) may be safe and effective enough to be used for repeated oocyte recovery from the same donor heifer[54]. Theoretically, one valuable heifer could produce several calves before even reaching puberty and many more over a lifetime[54]. However, the practical efficiency of this approach in a commercial setting needs to be determined. Some studies have shown that oocytes from heifers younger than six months may have reduced developmental competence owing to cytoplasmic immaturity[52]. It has been suggested (but not confirmed) that this could be overcome by gonadotropin stimulation before oocyte collection[54].

Nuclear transfer

Nuclear transfer is another technology with the potential to expedite both transgenic-animal production and bulk recombinant-protein manufacture. Donor nuclei from primary or cultured cells are transferred into enucleated oocytes, typically by fusing donor cells with enucleated oocytes. The fused cells are activated chemically or electrically to stimulate cell division in the resting oocytes and normal-looking blastocysts are transferred into hormonally synchronized recipient females for gestation. The resulting animals have the same nuclear genetic makeup as the source of the nucleus. Nuclear transfer directly from embryo cells has been used for more than a decade to clone live-born sheep[55,56], cattle[57,58], goats[59,60] and one pig[61].

If it can be made to work reproducibly on a large scale, nuclear transfer may dramatically increase the efficiency of transgenic production by quickly generating many transgenic offspring of a valuable founder animal. Female transgenic donor cells could be used to produce a founder 'mini-herd' of lactating production animals in one generation. In theory, large sets of identical animals may be produced in one step, although in practice surviving sets of five identical lambs[62] and seven identical calves[63] have been produced from nuclei derived from non-transgenic blastomeres. A set of eight identical non-transgenic calves was cloned from oviduct and epithelial cells of a single adult, although four of these died soon after birth[64].

Once a useful transgenic-animal line is identified, nuclear transfer could be used again for rapid herd expansion. Rather than waiting another generation for a single transgenic founder animal to mature and reproduce, the breeder would be able to generate a number of transgenic animals in a single breeding cycle. Donor nuclei may come from the cell line used to produce the original founders, from early transgenic embryos collected from founders that have been bred or from eggs collected from superovulated founder females and fertilized *in vitro*.

Animals created by nuclear transfer from groups of non-identical embryos, of course, would not be genetically identical to the founder or to each other but genetic identity *per se* is not required. The key is rapid reproduction of valuable transgenic animals for further characterization, development and/or recombinant-protein production. This approach may increase the number of useful animals produced at each stage, and more animals means more milk at each critical step of product development. Providing large quantities of recombinant protein sooner can accelerate the time to clinical trials by as much as one full generation, a time savings of 13–15 months for goats and two years for cows. This would translate into a significant potential advantage in a commercial setting by accelerating market launch and reducing time-related product-development costs.

A second key advantage of nuclear transfer for the transgenic production of biopharmaceuticals is the use of cultured transgenic cells as nuclear donors. Microinjection of transgene constructs into zygotes permits only gene insertion, and the transgene insertion site is usually random. Most of the cultured cells used to date for successful nuclear transfer have been embryo-derived cells or primary cell lines, which have limited growth capacity *in vitro*. The use of cultured cells that can be selected and clonally expanded *in vitro* would facilitate the development of transgenic animals with preselected gene insertion, deletions or modifications. Genetically transformed cells can be characterized *in vitro* and only clones with stably integrated transgenes would be used as nuclear donors. All of the animals created from such cells should be transgenic.

The feasibility of this strategy was first established with non-transgenic cultured cells. For example, lambs have been produced from embryo-derived cultured fibroblasts[65] and blastocyst-derived cell lines[56]. The first animal to be cloned from adult-derived cultured somatic cells, albeit with very low efficiency, was the lamb dubbed 'Dolly' and announced with much fanfare in 1997[66]. More recently, transgenic animals were produced by nuclear transfer from cultured fetal fibroblasts, including three identical lambs carrying human Factor IX[67] and three chimeric calves carrying marker genes[68]. A trio of identical female transgenic goats was cloned from a fetal somatic cell line derived from a transgenic fetus[60]. To date, germ-line transmission from these animals has not been reported.

Before they can make significant practical improvements in animal-production efficiency, nuclear-transfer methods will need to be improved to overcome technical problems such as low development and pregnancy rates, increased birth weight, nonspecific physical abnormalities, and perinatal demise. At least some of these may result from current *in vitro* manipulation methods[62]. Nevertheless, average efficiencies (the ratio of live births to transferred embryos) of 40% have been reported for cattle[54,58,63,69] and sheep[62], and one commercial operation produced 100 non-transgenic calves by nuclear transfer in one year[69]. Accelerated herd scale-up by nuclear transfer may be economically feasible, but this remains to be confirmed on a large scale for transgenic animals.

Conclusion

The transgenic production of recombinant proteins offers a safe, efficient and economical way to manufacture valuable biotherapeutics. A number of recombinant proteins have been produced in several transgenic animal species, and the first three transgenically produced recombinant proteins are in clinical trials. However, current methods of creating transgenic founder animals are still relatively inefficient and time consuming. Interesting technical challenges include developing better control over transgene timing and expression, and reducing the effective intergenerational time among early-stage transgenic production animals. New approaches are drawing on developments from molecular genetics and reproductive physiology to increase the efficiency of producing and reproducing useful founder animals. This work is likely to yield new insights into the basic biology of both the genes and the animals that express them.

References

1 Pharmaceutical Research and Manufacturers of America (1998) *Approved Biotechnology Drugs and Vaccines*, Pharmaceutical Research and Manufacturers of America, Washington, DC, USA
2 Simons, J. P. *et al.* (1988) *Biotechnology* 6, 179–183
3 Meade, H. M. *et al.* (1999) in *Gene Expression Systems: Using Nature for the Art of Expression* (Fernandez, J. M. and Hoeffler, J. M. eds), pp. 399–427, Academic Press
4 Cole, E. S., Higgins, E., Bernasconi, R., Garone, L. and Edmunds, T. (1994) *J. Cell Biochem.* 18D (Suppl.), 265
5 Rohricht, P. (1999) *BioPharm* 12, 46–49
6 Echelard, Y. (1996) *Curr. Opinion Biotechnol.* 7, 536–540
7 Wall, R. J., Kerr, D. E. and Bondioli, K. R. (1997) *J. Dairy Sci.* 80, 2213–2224
8 Colman, A. (1998) *Biochem. Soc. Symp.* 63, 141–147
9 Edmunds, T. *et al.* (1998) *Blood* 91, 4561–4571
10 Velander, W. H., Lubon, H. and Drohan, W. N. (1997) *Sci. Am.* 276, 70–74
11 Young, M. Y., Okita, W. B., Brown, E. M. and Curling, J. M. (1997) *BioPharm* 10, 34–38
12 Stokes, R. (1999) *Pharm. Technol. Eur.* 11, 58–59
13 Ziomek, C. A. (1996) in *Viral Safety and Evaluation of Viral Clearance from Biopharmaceutical Products* (Brown, F. and Lubiniecki, A. S., eds), pp. 265–268, Karger, Basel, Switzerland
14 Gavin, W. G. (1998) in *Transgenic Animals: Generation and Use* (Houdebine, L. M., ed.), pp. 19–21, Harwood Academic Publishers, Amsterdam, The Netherlands
15 Chan, A. W. S., Homan, E. J., Ballou, L. U., Burns, J. C. and Bremel, R. D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 14028–14033
16 Paleyanda, R. K. *et al.* (1997) *Nat. Biotechnol.* 15, 971–975
17 McKee, C. *et al.* (1998) *Nat. Biotechnol.* 16, 647–651
18 DiTullio, P. *et al.* (1992) *Biotechnology* 10, 74–77
19 Stromqvist, M. *et al.* (1997) *Transgenic Res.* 6, 271–278
20 Butler, S. P., vanCott, K., Subrumanian, A., Gwazdauskas, F. C. and Velander, W. H. (1997) *Thromb. Haemost.* 78, 537–542
21 Bawden, W., Passey, R. J. and Mackinlay, A. G. (1994) *Biotechnol. Genet. Eng. Rev.* 12, 89–137
22 Rijnkels, M., Kooiman, P. M., de Boer, H. A. and Pieper, F. R. (1997) *Mamm. Genome* 8, 148–152
23 Clark, A. J. (1998) *Biochem. Soc. Symp.* 63, 133–140
24 Drohan, W. N. *et al.* (1994) *Transgenic Res.* 3, 355–364
25 Colman, A. (1996) *Am. J. Clin. Nutr.* 63, 639S–645S

Targeted disruption of the α1,3-galactosyltransferase gene in cloned pigs

Yifan Dai[1]*, Todd D. Vaught[1], Jeremy Boone[1], Shu-Hung Chen[1], Carol J. Phelps[1], Suyapa Ball[1], Jeff A. Monahan[1], Peter M. Jobst[1], Kenneth J. McCreath[2], Ashley E. Lamborn[1], Jamie L. Cowell-Lucero[1], Kevin D. Wells[1], Alan Colman[2], Irina A. Polejaeva[1], and David L. Ayares[1]

Galactose-α1,3-galactose (α1,3Gal) is the major xenoantigen causing hyperacute rejection in pig-to-human xenotransplantation. Disruption of the gene encoding pig α1,3-galactosyltransferase (α1,3GT) by homologous recombination is a means to completely remove the α1,3Gal epitopes from xenografts. Here we report the disruption of one allele of the pig α1,3GT gene in both male and female porcine primary fetal fibroblasts. Targeting was confirmed in 17 colonies by Southern blot analysis, and 7 of them were used for nuclear transfer. Using cells from one colony, we produced six cloned female piglets, of which five were of normal weight and apparently healthy. Southern blot analysis confirmed that these five piglets contain one disrupted pig α1,3GT allele.

Galactose-α1,3-galactose (α1,3Gal) epitopes are a common carbohydrate structure on the cell surface of almost all mammals with the exception of humans, apes, and Old World monkeys[1]. Synthesis of the α1,3Gal epitope is catalyzed by the enzyme α(1,3)galactosyltransferase (α1,3GT)[2]. Humans do not have a functional copy of the α1,3GT gene, and hence do not show α1,3Gal surface expression. The presence of the α1,3Gal antigen on the surface of pig cells and tissues is the major cause of hyperacute rejection (HAR) in pig-to-human xenotransplantation[2-4]. It has been reported that, in humans, up to 1% of the total circulating IgG is anti-α1,3Gal natural antibody[5]. A number of strategies have been used to reduce or eliminate α1,3Gal-induced HAR. These methods[2-4] include overexpression of α2,3-sialyltransferase or α1,2-fucosyltransferase in pig cells to compete with α1,3GT; treatment of pig organs with α-galactosidase to remove surface α1,3Gal epitopes; expression of complement inhibitor genes, such as human decay-accelerating factor (DAF), in transgenic pig organs to suppress the complement reaction; and temporary depletion of natural anti-α1,3Gal antibody from recipients before transplantation. All these methods only partially or temporarily remove the α1,3Gal from the surface of the xenografts, however, and the residual α1,3Gal molecules are still sufficient to activate the complement cascade and cause destruction of the grafts[2-4]. Complete elimination of α1,3Gal epitopes from the donor organs should be achievable by removal of the α1,3GT gene. α1,3GT knockout mice have been made by a number of groups[6-7]. When tissues from these mice are exposed to human serum, they bind substantially less human anti-Gal xenoantibody than do tissues from normal mice, resulting in a significant decrease in human complement activation[6].

The cloning of sheep[8], goat[9], cattle[10], and pigs[11] by somatic cell nuclear transfer provides an alternative means of disrupting or deleting genes in mammals other than mice. The production of cloned sheep with targeted insertions at the ovine α1(I)-procollagen (*COL1A1*) locus showed that viable animals can be produced via nuclear transfer with gene-targeted cultured fibroblasts[12]. The α1,3GT gene has recently been successfully deleted in sheep fibroblasts and in fetuses cloned from targeted cells[13]. Although no viable animals resulted, these experiments showed that it is feasible to disrupt the α1,3GT gene using nuclear transfer techniques in livestock. Lai *et al.* have recently described the disruption of one allele of the α1,3GT gene in pig fibroblasts and in four live piglets cloned from these cells[14]. However, the only evidence of gene targeting offered in this report was PCR analysis of recombination junctions. Here we present genomic Southern blot analyses showing successful disruption of one copy of the α1,3GT gene in cultured male and female porcine fetal fibroblasts. To date we have produced five apparently healthy α1,3GT knockout female piglets by nuclear transfer.

Results and discussion

Because the α1,3GT gene is expressed well in porcine fetal fibroblasts (PPL Therapeutics, unpublished data), it is possible to enrich for homologous recombination events using a promoter-trap knockout-vector strategy[12]. Two similar knockout vectors, pPL654 and pPL657, were constructed from isogenic DNA of SLA1-10 and PCFF4-2 cells, respectively, by inserting an *IRES-neo*-poly A cassette into the 5′ end of exon 9 (Fig. 1A). Because the majority of the coding region of the pig α1,3GT gene, including the sequences encoding the catalytic domain, is located in exon 9, successful targeting using these vectors is expected to result in functional inactivation of the gene[6-7].

Four different early-passage (P2 or P3) primary porcine fetal fibroblasts cell lines were used for transfection: the male cell lines SLA1-10, PCFF4-2, and PCFF4-3 and the female cell line PCFF4-6. SLA1-10 cells were transfected with the isogenic vector pPL654, and PCFF4-2 cells were transfected with the isogenic vector pPL657. The PCFF4-3 and PCFF4-6 cell lines were derived from sibling fetuses of the fetus used to derive PCFF4-2, and therefore were transfected with the pPL657 vector. G418-resistant colonies were screened by 3′ PCR with neo442S (a sequence from the 3′ end of *neo*) and αGTE9A2 (a sequence from the 3′ end of exon 9 in sequences located outside the 3′ recombination

[1]*PPL Therapeutics Inc., 1700 Kraft Drive, Blacksburg, Virginia 24060, USA.* [2]*PPL Therapeutics Ltd., Roslin, Midlothian EH25 9PP, UK.*
**Corresponding author (ydai@ppl-therapeutics.com).*

of these cells with the pPL657 GT knockout vector. When compared with PCFF4-2 cells, PCFF4-6 cells had a very similar knockout efficiency even though they were transfected with vector made from non-isogenic DNA. As both the PCFF4-2 and PCFF4-6 cell lines were derived from sibling fetuses of the same pregnancy, it is possible that they share a common allele.

We used seven Southern blot–confirmed α1,3GT-knockout single colonies for nuclear transfer. All cells used for nuclear transfer were from the aliquots that had been frozen immediately after the initial 3′ PCR screening. The karyotype of each colony was checked; all had chromosome numbers in a range similar to that of freshly isolated porcine fetal fibroblast cells (~70% of spreads with 38 chromosomes). On average, approximately 150 reconstructed nuclear-transfer embryos were transferred to the oviducts of each estrus-synchronized recipient female. All seven colonies used for nuclear transfer resulted in very high initial pregnancy rates at day 25 (50%–86%) (Table 2). However, all pregnancies established from colonies 657A-A8 and 657A-F12 were lost between days 25 and 45. In contrast, the other five colonies resulted in pregnancy rates in excess of 50% at day 45.

One spontaneously aborted day 38 fetus from a 657A-A8 nuclear transfer recipient was recovered. LR-PCR and Southern blot analysis confirmed that the fetus contained a disrupted α1,3GT locus (data not shown). Southern blot results from 657A-I11 cells showed that the 9-kb knockout band was less intense than the 7-kb endogenous α1,3GT band, indicating that this was most likely a mixed colony containing both wild-type and heterozygous knockout cells. There was some concern that fetuses derived from wild-type cells in the mixed colony could affect the development of, or outcompete, *in utero* fetuses derived from the α1,3GT-knockout cells. To test this, we terminated by hysterectomy a day 32 pregnancy derived from nuclear transfer with 657A-I11 cells. Seven fetuses were recovered, of which six were of normal size and one substantially smaller. Southern blot analysis showed that six of the seven fetuses contained a disrupted α1,3GT locus (Fig. 2A). Notably, it was the smaller fetus (fetus no. 2) that was wild type, and all six normal-sized fetuses contained an α1,3GT knockout allele. These results suggested that there was no discrimination against the heterozygous α1,3GT knockout fetuses *in utero*.

Six live piglets derived from the 657A-I11 cells were born on December 25, 2001. Five were of normal size and weight (Fig. 3); one (no. 2) was stunted, weighing less than 1 pound. Southern blot analysis indicated that five of the six offspring were α1,3GT heterozygous knockouts (Fig. 2B). Again, the one negative (wild-type) piglet was the underdeveloped runt. These results, when considered along with the analysis of the seven day 32 fetuses from 657A-I11 cells, suggested that the colony 657A-Ill was indeed a mixed population contaminated with wild-type cells. All fetuses and offspring obtained from the α1,3GT knockout cells in the 657A-I11 population were developmentally normal. Physical examination of the five knockout piglets at one month of age found no abnormalities. This contrasts with the report by Lai *et al.*[14], in which only four of seven piglets survived for more than a month and three of the surviving piglets had mild physical abnormalities. These differing outcomes may have been due to many factors, including different pig breeds, different condition of cells used for nuclear transfer, and different embryo manipulation methods.

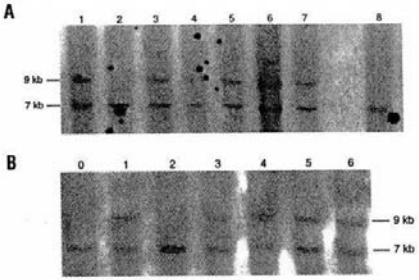

Figure 2. Southern blot analysis of α1,3GT gene knockout fetuses and piglets. (A) Southern analysis of DNA from seven day 32 fetuses. Lane 1–7 are *Bst*EII-digested genomic DNA from seven day 32 fetuses derived from 657A-I11 cells. Lane 8 contains normal pig DNA digested with *Bst*EII as a negative control. The 7-kb band represents the endogenous α1,3GT gene and the 9-kb band the disrupted α1,3GT locus. (B) Southern analysis of DNA from six piglets. Lane 0 is the normal pig DNA digested with *Bst*EII. Lanes 1–6 are *Bst*EII-digested DNA from six piglets cloned from 657A-I11 cells. The 7-kb band represents the endogenous α1,3GT gene and the 9-kb band represents the disrupted α1,3GT locus.

We have produced apparently healthy heterozygous α1,3GT knockout piglets by nuclear transfer. We have an additional 16 ongoing second- and third- trimester pregnancies beyond day 45 from two female and three male α1,3GT knockout colonies (Table 2). We expect that most will go to term as we have never lost any pregnancies of cloned pigs after 45 days of gestation (data not shown). The next step is to obtain homozygous pigs with both α1,3GT alleles inactivated. This could be done either through natural breeding of male and female heterozygous knockout animals or through gene targeting with heterozygous knockout cells to disrupt the second α1,3GT allele before a second round of nuclear transfer. As we already have six lines of early-passage heterozygous α1,3GT gene–disrupted fetal fibroblasts, obtained from the day 32 657A-I11 fetuses, it will probably be considerably faster to create the second knockout in these cells *in vitro* and obtain homozygous knockout animals by nuclear transfer. Natural breeding to homozygosity will also be used, but this method will take considerably longer because of the gestation time of the male knockout clones *in utero* and the time to sexual maturity.

Live births have been reported in cattle from recloning experiments that used fibroblasts obtained from cloned fetuses[17]. Recloning experiments by our group, using wild-type porcine fetal fibroblasts derived from a day 40 cloned fetus, have shown an 80% pregnancy rate at day 45 (PPL Therapeutics, unpublished data). These data suggest that it will be feasible to obtain homozygous α1,3GT knockout pigs by a second knockout and recloning

Table 2. Summary of nuclear transfer results from α1,3GT knockout primary fibroblast cells

Nuclear donor cell line	PCFF4-6	PCFF4-6	PCFF4-6	PCFF4-6	PCFF4-2	PCFF4-2	PCFF4-3
Cell clone	657A-I11	657A-A8	657A-F12	657A-I6	657F-J10	657F-C11	657B-K8
Sex	F	F	F	F	M	M	M
Embryos transferred to recipients	1097	825	591	976	1009	775	1105
Recipients	7	5	4	6	6	4	7
Pregnancies at day 25	6	3	2	5	5	2	6
Pregnancies at day 45	3[a]	0	0	4	4	2	4[b]
Piglets at birth[c]	6	0	0	Pending	Pending	Pending	Pending

[a]Based on 6 recipients since one day 32 pregnancy was terminated for fetal fibroblast isolation. [b]Based on 6 recipients since one day 39 pregnancy was terminated for fetal fibroblast isolation. [c]As dated on January 25, 2002. Six piglets were born from one 657A-I11 recipient on December 25, 2001. Another 16 recipients beyond day 45 of pregnancy are due after January 25, 2002.

Figure 3. Five α1,3GT gene knockout piglets at 2 weeks of age.

strategy. In mice, the homozygous knockout of α1,3GT gene is not an embryonic lethal mutation, although such mice have developed cataracts[6]. Pig cells express significantly more α1,3Gal epitopes on their surface than do mouse cells, and it has therefore been proposed that α1,3GT may have some additional, unknown role in pigs[18]. Although heterozygous α1,3GT knockout pigs are developmentally normal, it is not known if complete deletion of both alleles of α1,3GT will be more (or less) problematic in pigs than in mice.

Complete removal of the α1,3Gal epitope, the major xenoantigen, combined with transgenic expression of complement regulatory proteins should prevent the hyperacute rejection of pig xenografts even in the presence of a low background of non-α1,3Gal xenoantigens. The success of xenotransplantation will also depend on risk assessment of safety factors such as porcine endogenous retroviruses and on the development of strategies that address delayed vascular and T-cell-mediated rejection. Together, these approaches may provide a near-term solution to the chronic shortage of human organs (such as heart and kidneys) and valuable tissues such as insulin-producing islet cells.

Experimental protocol

Isolation and transfection of primary porcine fetal fibroblasts. PCFF4-1 to PCFF4-10 fetal fibroblast cells were isolated from 10 fetuses of the same pregnancy at day 33 of gestation. After removing the head and viscera, fetuses were washed with Hanks' balanced salt solution (HBSS; Gibco-BRL, Rockville, MD), placed in 20 ml of HBSS, and diced with small surgical scissors. The tissue was pelleted and resuspended in 50-ml tubes with 40 ml of DMEM and 100 U/ml collagenase (Gibco-BRL) per fetus. Tubes were incubated for 40 min in a shaking water bath at 37°C. The digested tissue was allowed to settle for 3–4 min and the cell-rich supernatant was transferred to a new 50-ml tube and pelleted. The cells were then resuspended in 40 ml of DMEM containing 10% fetal calf serum (FCS), 1× nonessential amino acids, 1 mM sodium pyruvate (Gibco-BRL), and 2 ng/ml basic fibroblast growth factor (bFGF; Roche Molecular Biochemicals, Indianapolis, IN) and seeded into 10-cm dishes. All cells were cryopreserved upon reaching confluence. SLA1-1 to SLA1-10 cells were isolated from 10 fetuses at day 28 of pregnancy. Fetuses were mashed through a 60-mesh metal screen (Sigma, St. Louis, MO) using curved surgical forceps slowly so as not to generate excessive heat. The cell suspension was then pelleted and resuspended in 30 ml of DMEM containing 10% FCS, 1× nonessential amino acids, 2 ng/ml bFGF, and 10 μg/ml gentamycin. Cells were seeded in 10-cm dishes, cultured one to three days, and cryopreserved. For transfections, 10 μg of linearized vector DNA was introduced into 2 million cells by electroporation. Forty-eight hours after transfection, the transfected cells were seeded into 48-well plates at a density of 2,000 cells per well and were selected with 250 μg/ml of G418 (Gibco-BRL).

Knockout vector construction. Two α1,3GT knockout vectors, pPL654 and pPL657, were constructed from isogenic DNA of two primary porcine fetal fibroblasts, SLA1-10 and PCFF4-2 cells. A 6.8-kb α1,3GT genomic fragment, which includes most of intron 8 and exon 9, was generated by PCR from purified DNA of SLA1-10 cells and PCFF4-2 cells, respectively. The unique *Eco*RV site at the 5′ end of exon 9 was converted into a *Sal*I site and a 1.8-kb *IRES-neo*-poly A fragment was inserted into the *Sal*I site. IRES (internal ribosome entry site) functions as a translation initial site for neo protein. Thus, both vectors have a 4.9-kb 5′ recombination arm and a 1.9-kb 3′ recombination arm (Fig. 1A).

3′PCR and long-range PCR. Approximately 1,000 cells were resuspended in 5 μl embryo lysis buffer (ELB) (40 mM Tris, pH 8.9, 0.9% Triton X-100, 0.9% Nonidet P-40, 0.4 mg/ml proteinase K), incubated at 65°C for 15 min to lyse the cells, and heated to 95°C for 10 min to inactivate the proteinase K. For 3′ PCR analysis, fragments were amplified using the Expand High Fidelity PCR system (Roche Molecular Biochemicals) in 25 μl reaction volume with the following parameters: 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. For LR-PCR, fragments were amplified by using TAKARA LA system (Panvera/Takara, Madison, WI) in 50 μl reaction volume with the following parameters: 30 cycles of 10 s at 94°C, 30 s at 65°C, 10 min + 20 s increase/cycle at 68°C; and one final cycle of 7 min at 68°C. 3′ PCR and LR-PCR conditions for purified DNA was same as for cells except that 1 μl of purified DNA (30 μg/ml) was mixed with 4 μl ELB.

Southern blot analysis of cell samples. Approximately 10^6 cells were lysed overnight at 60°C in lysis buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% (w/v) Sarcosyl, 1 mg/ml proteinase K) and the DNA precipitated with ethanol. The DNA was then digested with *Bst*EII and separated on a 1% agarose gel. After electrophoresis, the DNA was transferred to a nylon membrane and probed with the 3′-end digoxigenin-labeled probe. Bands were detected using a chemiluminescent substrate system (Roche Molecular Biochemicals).

Southern blot analysis of pig tissues. Fetal tissues and piglet tails were lysed overnight at 60°C in a shaking incubator with approximately 1 ml lysis solution (50 mM Tris, pH 8.0, 0.15 M NaCl, 10 mM EDTA, 1% SDS, 25% sodium perchlorate, 1% 2-mercaptoethanol, and 200 μg/ml proteinase K) per 175 mg tissue. DNA was subjected to phenol/chloroform extraction and precipitated with isopropyl alcohol. Resolubilized DNA was treated with RNase A (1 mg/ml) and RNase T1 (1,000 U/μl) at 37°C for 1 h, with proteinase K (20 mg/ml) at 55°C for 1 h, then extracted with phenol/chloroform, precipitated with ethanol, and resuspended in TE buffer. About 10 mg DNA was digested with *Bst*EII and separated on an 1% agarose gel. Following electrophoresis, the DNA was transferred to a nylon membrane and probed with the 3′-end digoxigenin-labeled probe. Bands were detected using a chemiluminescent substrate system.

Nuclear transfer procedure. Enucleation of *in vitro*–matured oocytes (BioMed, Madison, WI) was begun between 40 and 42 h post-maturation as described previously[11]. A single fibroblast cell was placed under the zona pellucida in contact with each enucleated oocyte. Fusion and activation were induced by application of an AC pulse of 5 V for 5 s followed by two DC pulses of 1.5 kV/cm for 60 μs, each using an ECM2001 Electrocell Manipulator (BTX Inc., San Diego, CA). Fused embryos were cultured in NCSU-23 medium for 1–4 h at 38.6°C in a humidified atmosphere of 5% CO_2, and then transferred to the oviduct of an estrus-synchronized recipient gilt. Crossbred gilts (large white/Duroc/landrace) (280–400 lbs) were synchronized as recipients by oral administration of 18–20 mg Regu-Mate (Altrenogest, Hoechst, Warren, NJ) mixed into their feed. Regu-Mate was fed for 14 consecutive days. Human chorionic gonadotropin (hCG, 1,000 units; Intervet America, Millsboro, DE) was administered intramuscularly 105 h after the last Regu-Mate treatment. Embryo transfers were done 22–26 h after the hCG injection.

Acknowledgments

We thank B. Gragg, W. Lucero, T. Akers, H. Bishop, and J. McPherson for technical contributions to embryo transfer and animal husbandry; A. Garst and J. Hencke for help in nuclear transfer; C. Koike and T. E. Starzl at the University of Pittsburgh for providing us with porcine α1,3GT gene genomic sequence; M. Moore, A. Kind, and A. Schnieke for valuable contributions in discussions; and staff at the Virginia Maryland Regional Veterinary College for prenatal care of some of the nuclear transfer recipients and physical examination of piglets. This research was funded in part by a grant from the National Institute of Standards and Technology (NIST) Advanced Technology Program (ATP).

Competing interests statement

The authors declare that they have no competing financial interests.

Received 8 January 2002; accepted 22 January 2002

1. Galili, U., Shohet, S.B., Kobrin, E., Stults, C.L. & Macher, B.A. Man, apes, and Old World monkeys differ from other mammals in the expression of α-galactosyl epitopes on nucleated cells. *J. Biol. Chem.* **263**,17755–17762 (1988).
2. Joziasse, D.H. & Oriol, R. Xenotransplantation: the importance of the Galα1,3Gal epitope in hyperacute vascular rejection. *Biochim. Biophys. Acta* **1455**, 403–418 (1999).
3. Galili, U. The α-Gal epitope (Galα1-3Galβ1-4GlcNAc-R) in xenotransplantation. *Biochimie* **83**, 557–563 (2001).
4. Sandrin, M.S. & McKenzie, I.F.C. Galα(1,3)Gal, the major xenoantigen(s) recognised in pigs by human natural antibodies. *Immunol. Rev.* **141**, 169–190 (1994).
5. Galili, U., Macher, B.A., Buehler, J. & Shohet, S.B. Human natural anti-α-galactosyl IgG. II. The specific recognition of α(1-3)-linked galactose residues. *J. Exp. Med.* **162**, 573–582 (1985).
6. Tearle, R.G. *et al.* The α-1,3-galactosyltransferase knockout mouse. Implications for xenotransplantation. *Transplantation* **61**, 13–19 (1996).
7. Thall, A.D., Maly, P. & Lowe, J.B. Oocyte Galα1,3Gal epitopes implicated in sperm adhesion to the zona pellucida glycoprotein ZP3 are not required for fertilization in the mouse. *J. Biol. Chem.* **270**, 21437–21440 (1995).
8. Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. & Campbell, K.H.S. Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–813 (1997).
9. Baguisi, A. *et al.* Production of goats by somatic cell nuclear transfer. *Nat. Biotechnology* **17**, 456–461 (1999).
10. Cibelli, J.B. *et al.* Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280**, 1256–1258 (1998).
11. Polejaeva, I.A. *et al.* Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* **407**, 86–90 (2000).
12. McCreath, K.J. *et al.* Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature* **405**, 1066–1069 (2000).
13. Denning, C. *et al.* Deletion of the α(1,3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep. *Nat. Biotechnol.* **19**, 559–562 (2001).
14. Lai, L. *et al.* Production of α-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science* **295**, 1089–1092 (2002).
15. Kim, H.S., Popovich, B.W., Shehee, W.R., Shesely, E.G. & Smithies, O. Problems encountered in detecting a targeted gene by the polymerase chain reaction. *Gene* **103**, 227–233 (1991).
16. Shuldiner, A.R., Tanner, K., Moore, C.A. & Roth, J. RNA template-specific PCR: an improved method that dramatically reduces false positives in RT-PCR. *Biotechniques* **11**, 760–763 (1991).
17. Zakhartchenko, V. *et al.* Nuclear transfer in cattle with non-transfected and transfected fetal or cloned transgenic fetal and postnatal fibroblasts. *Mol. Reprod. Dev.* **60**, 362–369 (2001).
18. Tanemura, M., Maruyama, S. & Galili, U. Differential expression of α-Gal epitopes (Galα1-3Galβ1-4GlcNAc-R) on pig and mouse organs. *Transplantation* **69**, 187–190 (2000).

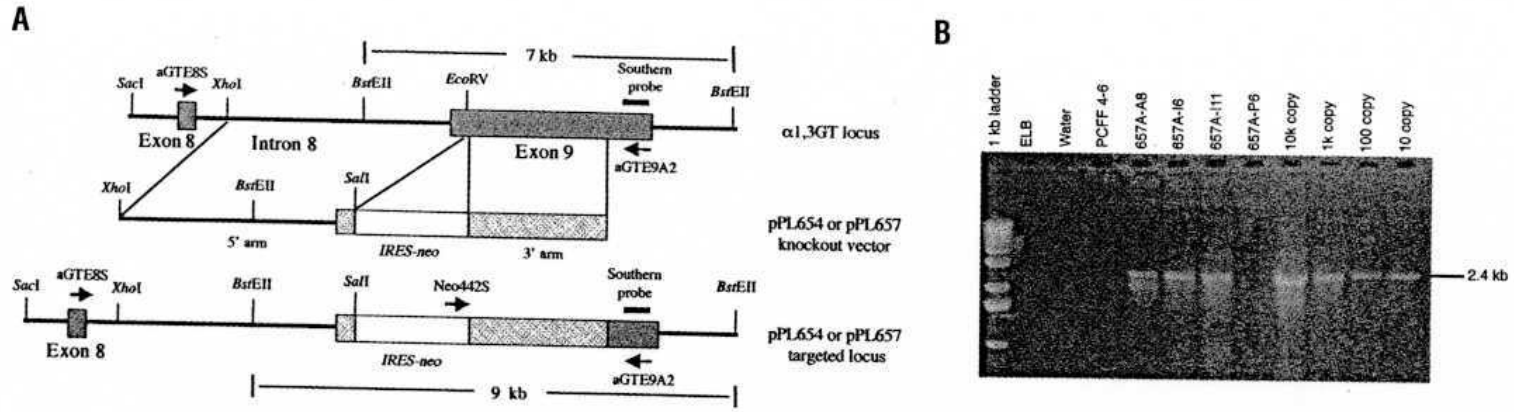

Figure 1. Disruption of the porcine α1,3GT gene by gene targeting. (A) Diagram of the porcine α1,3GT locus, the corresponding α1,3GT genomic sequences used as 5′ and 3′ arms in the α1,3GT knockout vectors, and the structure of the targeted locus after homologous recombination. The two α1,3GT knockout vectors (pPL654 and pPL657) have the same design except that the α1,3GT sequences present in the recombination arms are derived from different cells. The names and positions of the primers used for 3′ PCR and long-range PCR are indicated by short arrows. Neo442S: 5′-CATCGCCTTCTATCGCCTTCTT-3′; αGTE8S: 5′-AGAGGTCGTGACCATAACCAGAT-3′; αGTE9A2: 5′-AGCCCATCGTGCTGAACATCAAGTC-3′. The short bar indicates the probe used for α1,3GT Southern blot analysis. The predicted size of Southern hybridization bands with *Bst*EII digestion, for both the endogenous α1,3GT locus and the α1,3GT targeted locus, is indicated. (B) 3′ PCR results. Approximately 1,000 cells were used for 3′ PCR, with Neo442S as the forward primer and αGTE9A2 as the reverse primer. Three negative controls, embryo lysis buffer (ELB), water, and wild-type (PCFF4-6) cells, were used to ensure that the PCR reaction was not contaminated. 657A-A8, 657A-I6, 657A-I11, and 657A-P6 are individual G418-resistant colonies. A positive-control plasmid was included, at dilutions from 10,000 copies down to 10 copies as indicated. (C) LR-PCR results. Approximately 1,000 cells were used, with αGTE8S as the forward primer and αGTE9A2 as the reverse primer. ELB, water, and pCFF4-6 cells were used as negative controls. 657A-A8, 657A-I6, 657A-I11, and 657A-P6 are individual G418-resistant colonies.

Table 1. Summary of 3′ PCR, LR-PCR, and Southern analysis results of G418-resistant colonies

Cells (sex)	Knockout vectors	No. of G418^R colonies	No. of 3′ PCR$^+$ colonies (%)	No. of LR-PCR$^+$ colonies(%)	No. of Southern$^+$ colonies(%)
SLA1-10 (M)	pPL654	127	4 (3%)	0	0
PCFF4-2 (M)	pPL657	179	22 (12%)	11 (6%)	2 (1%)
PCFF4-3 (M)	pPL657	200	5 (2.5%)	1 (0.5%)	1 (0.5%)
PCFF4-6 (F)	pPL657	599	69 (11.5%)	18 (3%)	14 (2%)

Results for SLA1-10, PCFF4-2, and PCFF4-6 cells are from two individual transfections for each cell; result for PCFF4-3 cells is from one transfection.