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Review

Lentiviral vectors: are they the future of animal transgenesis?

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Park F. Lentiviral vectors: are they the future of animal transgenesis? *Physiol Genomics* 31: 159–173, 2007. First published August 7, 2007; doi:10.1152/physiolgenomics.00069.2007.—Lentiviral vectors have become a promising new tool for the establishment of transgenic animals and the manipulation of the mammalian genome. While conventional microinjection-based methods for transgenesis have been successful in generating small and large transgenic animals, their relatively low transgenic efficiency has opened the door for alternative approaches, including lentiviral vectors. Lentiviral vectors are an appealing tool for transgenesis in part because of their ability to incorporate into genomic DNA with high efficiency, especially in cells that are not actively dividing. Lentiviral vector-mediated transgene expression can also be maintained for long periods of time. Recent studies have documented high efficiencies for lentiviral transgenesis, even in animal species and strains, such as NOD/*scid* and C57Bl/6 mouse, that are very difficult to manipulate using the standard transgenic techniques. These advantages of the lentiviral vector system have broadened its use as a gene therapy vector to additional applications that include transgenesis and knockdown functional genetics. This review will address the components of the lentiviral vector system and recent successes in lentiviral transgenesis using both male- and female-derived pluripotent cells. The advantages and disadvantages of lentiviral transgenesis vs. other approaches to produce transgenic animals will be compared with regard to efficiency, the ability to promote persistent transgene expression, and the time necessary to generate a sufficient number of animals for phenotyping.

lentiviral vector; integration; progeny; transgenic animals

TRANSGENIC TECHNOLOGIES have emerged as invaluable tools to manipulate the genome in biomedical, veterinary, and agricultural research. Since the early work by Brackett et al. (6) using SV40 DNA transfection into rabbit spermatozoa and the subsequent work by Jaenisch and colleagues using wild-type retroviruses (54, 55, 57) to manipulate embryonic stem cells, there has been considerable interest in developing various tools and approaches to modify the genome to produce transgenic animals. Presently, the most commonly accepted method to produce transgenic animals remains the pronuclear injection of plasmid DNA into fertilized oocytes, first developed by Gordon et al. (35). This method has been successful in generating a wide variety of small (mice and rats) (31, 35, 126) and large transgenic mammals (pigs, sheep, rabbits, goats, and cattle) (5, 24, 40). Unfortunately, this method is still hampered by a relatively low efficiency (40, 46, 94), particularly in species other than mice (46, 94).

This is a considerable challenge to overcome because of the importance of these other species for studying biological function and developing new biomedical treatments in humans. Although the inefficiencies of pronuclear injections can be overcome using small transgenic animals by high-throughput screening for DNA integration, this type of approach becomes economically more challenging in larger animals, such as sheep, goats, or cows, because of the extreme costs associated with this procedure ($60,000–$300,000) (135).

For this reason, new strategies that enhance the production and variety of transgenic animals would be valuable and an important asset to the scientific community. One emerging technique has been the use of lentiviral vectors, which were initially developed for gene therapy to correct genetic disorders in somatic cells (91, 109). This review will examine recent work using lentiviral vectors to generate transgenic animals and discuss the various pluripotent cell types that may be used in the context of lentiviral transgenesis. Comparisons with previous work regarding the use of nonviral and retroviral vectors in transgenic protocols will be discussed to provide a historical perspective on the advantages and disadvantages of the lentiviral transgenic approach.

VECTORS FOR TRANSGENESIS: NUCLEIC ACIDS AND VIRUSES

Nucleic Acid-Based Vectors

For most applications, plasmid DNA has been used to create transgenic animals. Its basic composition is a sequence of DNA that contains one or more antibiotic-resistant genes (ampicillin, neomycin, puromycin, etc.) and a bacterial origin of replication. Following the insertion of an expression cassette, the genetically engineered plasmid DNA can be propagated in bacteria under antibiotic selection and purified for

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direct use to inject or transfect mammalian cells. In general, the insert is removed from the vector, which has been thought to hamper the integration of the DNA. Table 1 shows a comparison of the factors involved in the plasmid DNA microinjection approach compared with the lentiviral vector system (which will be discussed below).

The number of copies of plasmid DNA that can be integrated varies from one to several hundred copies, which can be found as concatemers with a predominant head-to-tail orientation (3, 4, 77). Inhibitory effects from nearby genomic areas can alter the expression of the transgene (1), which is why larger genomic fragments are often used that include insulator sequences to maximize the production of transgene-expressing transgenic animals (113). The unrestricted size limitation is the single most important advantage to creating transgenic animals through direct incorporation of plasmid DNA. Even extremely large genomic DNA pieces have been cloned into yeast artificial chromosomes (YAC; 1- to 2-Mb insert size) or bacterial/P1-derived artificial chromosomes (BAC/PAC; <300-kb insert size) before transgenic applications. The details regarding the construction and advantages of artificial chromosomes have been reviewed elsewhere (33). However, there can be problems with the use of YAC/BAC/PAC because of rearrangements, shearing, and the technical nature of handling large constructs.

Nucleic acids generally integrate randomly (113), but there have been several attempts to achieve direct integration into more discrete sites. Hollis et al. (50) co-injected a donor plasmid DNA and phage ΦC31 integrase mRNA into the pronucleus to demonstrate site-specific integration by the plasmid DNA during the generation of transgenic mice. Although these investigators were able to produce a transgenic mouse, only 1 of 105 (or <1%) of the pups was found to be transgenic (50). This study illustrates that site-specific integration is possible, but further investigation is warranted to enhance the efficiency of this approach. Moreover, it remains to be determined whether this integration site is sensitive or resistant to epigenetic modifications that affect gene expression.

Even with the disadvantage of a low transgenic efficiency using pronuclear microinjection, this approach still remains the most simple and least labor-intensive routine method to produce transgenic animals, particularly in mice.

Integrating Viral Vectors: Lentiviral Vectors

There are a plethora of viral vector systems that have been developed for gene therapy applications, but the vast majority of these vectors are nonintegrating, which precludes their usefulness for transgenesis. The best characterized integrating viral vectors originate from the *retroviridae* family, the members of which are ideal for the genetic manipulation of mammalian cells because of their intrinsic ability to integrate into genomic DNA. At present, the use of simple retroviruses based on murine Moloney leukemia virus (MLV) (61, 92, 133) is becoming overshadowed by more sophisticated lentiviral vector systems that have superior efficiency in modifying cells in vitro and in vivo compared with MLV-based vectors. For this reason, the main topic of discussion will focus on the lentiviral vector system and its potential role in transgenesis.

The genetic composition and the applications for transgenesis of complex retroviral (or lentiviral) vectors are illustrated in Fig. 1. The lentivirus genome derived from immunodeficiency viruses, such as human immunodeficiency virus-1 (HIV-1), has been split into multiple fragments to minimize the potential formation of replication-competent viruses. These components are broken into the following categories: *1*) transfer or integrating vector, *2*) structural and packaging, and *3*) envelope plasmids. Since the transfer (integrating) vector plasmid is the only component that is transferred to the transgenic animal, the molecular composition of this plasmid will be described in this section, while the details regarding the packaging/structural and pseudotyping plasmids and detailed pro-

Table 1. *Comparison of nucleic acid vs. lentiviral vectors for animal transgenesis*

	Nucleic Acid (DNA)	Lentiviral Vector
Plasmid/vector preparation*	Minimal	Labor intensive
Packaging capacity	<50 kb (plasmid) <1 Mb (BAC/PAC) <1–2 Mb (YAC)	Most effective <10 kb
Site-specific gene targeting	Possible	Unlikely
Sites of integration	Random	Fairly random, but prefers active transcription units
Embryo manipulation skill	High	Low-to-moderate
Embryo survival	Low-to-high	High (>70%)
%Born transgenic/implanted embryo	Very low (~1–2%)	Low-to-moderate (8–50%)
Proportion born transgenic	Low (1–20%)†	Moderate-to-high§
Integrated transgene copies	Low (1–5 for large transgenes) High (>5–10 for small transgenes)	Single-to-multiple
Individual or multiple copies following integration	Concatemers	Individual
Expressing founders	~50%	High (at least 60%, but generally >90%)
Expressing F_1 progeny	Moderate (~50%)	Moderate (>50%)
Efficiency of cell types modified		
Zygote	Low-to-moderate‡	Extremely high
Sperm	Extremely low	Moderate-to-high
Germline stem cells	Low	Moderate-to-high
Somatic cells	Extremely low	Low-to-moderate

*Includes DNA preparation and other procedures that are required before embryo manipulation. †Species-dependent efficiency (1–10% in rats and livestock and 5–20% in mice). ‡Low efficiency in rats and large species (cows, chickens, pigs, etc.); moderate in mice. §Moderate in rats (>28%) and high in mice (>63%), pigs (>69%), and cows (>50%).

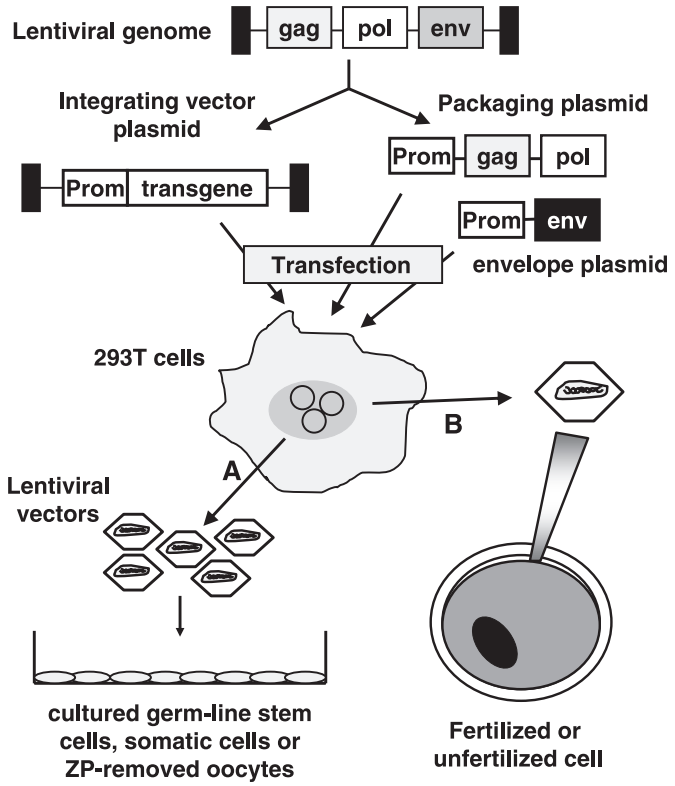

Fig. 1. Lentiviral vector production. The wild-type lentiviral genome is made up of 3 main genes (*gag*, *pol*, and *env*) and it is surrounded by long-terminal repeats (black color). The vector system is a split-genome format whereby the packaging and structural genes have been separated from the portion of the virus that promotes integration into the host genome. The *env* gene can be replaced with a heterologous glycoprotein from various viruses, including the prominently used VSV-G. The 3 plasmids (or more) are transfected into a cell line (293T cells) to produce the viral vectors, which are collected in the supernatant for in vitro transgenic applications either by infection (*A*) or by direct injection (*B*). Prom, viral or cellular promoter; ZP, zona pellucida.

tocols used to create the infectious lentiviral vector particles can be found elsewhere (18, 19, 61, 104, 130).

There are two main regions within the transfer plasmid that express the viral RNA genome following transfection during vector production: *1*) a multiple cloning site for the insertion of various expression cassettes and *2*) flanking long-terminal repeats (LTR) that have several distinct functions. First, the 5′-LTR can act like an RNA pol II promoter. Second, the 3′-LTR acts to terminate transcription and promote polyadenylation. Third, the LTR has recognition sequences necessary for integration into the genome. The RNA vector genome is ultimately reverse transcribed and integrated into the genomic DNA as a provirus using viral proteins obtained from the structural and packaging plasmids in trans during vector production.

Two important *cis*-acting DNA elements, the central polypurine tract sequence (cppt) and the woodchuck postregulatory element (WPRE), are also included in the transfer (integrating) vector to enhance the transduction efficiency and transcript stability, respectively (32, 99, 143, 145). The cppt is a small DNA fragment found in the *pol* gene of HIV that is usually cloned 5′ to the internal promoter region, whereas the WPRE is cloned 3′ to the inserted transgene so that it is in close proximity to the poly(A) signal in the 3′-LTR. Another important feature in the lentiviral transfer plasmid is a 400-bp deletion in the U3 region of the 3′-LTR, which debilitates the 5′-LTR RNA pol II promoter activity following integration (99, 146).

For the purposes of transgenesis, the primary benefit in using the lentiviral vector system is its efficient ability to integrate into the host genome (91, 100). However, proliferative stimuli before the administration of the lentiviral vectors or cells actively undergoing division have been shown to significantly enhance lentiviral vector transduction efficiency (93, 100–102). The latter characteristic could be a distinct advantage for lentiviral transgenesis compared with pronuclear microinjection by allowing for more rapid integration into the genome and reducing the potential for genetic mosaicism. A comparison between the lentiviral vector system and the pronuclear microinjection method can be found in Table 1.

TECHNIQUES USED IN THE MANIPULATION OF MALE- AND FEMALE-DERIVED EARLY PLURIPOTENT CELLS IN TRANSGENIC ANIMAL PRODUCTION

Both male- and female-derived cells have been used to generate transgenic animals. The differences in the approach being used with lentiviral vectors will be summarized and placed into context for each cell type.

Female-Derived Cells

In this section, the classic nucleic acid-based pronuclear injection approach will be discussed and compared with more modern approaches using lentiviral vectors. In general, fertilized zygotes have been manipulated at the single cell stage as well as unfertilized oocytes.

Pronuclear microinjection. This is the classic method to generate transgenic animals, and few changes have been made in the pronuclear injection method using DNA as the vector since the pioneering studies by Gordon et al. (35). The details of this approach have been extensively reviewed elsewhere (12, 113, 134). This procedure involves the transferring of genetic material by microinjecting DNA into the pronucleus of fertilized one-cell eggs, which are subsequently implanted into the oviduct of pseudopregnant surrogate females following mating with a vasectomized male. This method remains the predominant approach to produce transgenic animals because of its proven track record in generating a variety of transgenic animals, including mice, rats, pigs, chickens, cows, goats, and rabbits (5, 24, 31, 35, 40, 126).

Even with the successes described for this method, it has been fraught with a low transgenic efficiency. Embryo survival following microinjection is species dependent. It ranges from low viabilities in cows (17%) (14) to more moderate-to-high survival rates in rats and mice (31–87%) (7, 31, 126). In addition, integration of the constructs is often poor, and the generation of transgenic founder rats and other large animals, including cows and pigs, averages from 1 to 5% (7, 31, 126), whereas transgenic founder mice can be generated at efficiencies ranging from 5 to 20% (7, 134). In general, there is an ~50% probability that any given transgenic line will express the transferred gene product (134). It is important to note that the production of founder transgenic mice has been shown to be strain dependent, and that certain inbred strains of mice, such as C57Bl/6, can have an extremely low efficiency (0.5–

Table 2. *Summary of lentiviral vector applications using female and male pluripotent cells*

Species	Promoter	Transgene	%Embryo Viability (No. of Viable Treated Cells/Total Cells)	%Born (No. of Birth/ Transferred Embryos)	No. of F_0 Transgenic/ Total Examined (Transgene Expression)	F_0 Vector Copy No.	No. of F_1 Transgenic/Total Examined (Transgene Expression)	Method	Ref. No(s).
				Female cells					
Rat	UbC	GFP	90% (210/233)	17% (22/130)	13/22 (9)	1–7	NP	PVZ	Ref. 74
Rat	UbC	GFP	57% (65/114)	43% (28/65)[a]	13/28 (12)	1–4	18/64 (18)	PVZ	Ref. 131
Rat	CAG	GFP	100% (121/121)	50% (60/121)	13/60	1–4	68/139	PVZ	Ref. 81
Rat	SYN	GRIP1	40% (20/50)	55% (11/20)	5/11 (2)	>1	15/44 (9)	PVZ	Ref. 90
Rat	U6	DAZL shRNA	NP	NP	10/75 (6)	N/D	33/400	PVZ	Ref. 21
Mouse	UbC	GFP	86% (231/270)	21% (92/446)	63/73 (58)	1–21	NP	PVZ	Ref. 74
Mouse	MH	GFP	81% (86/106)	15% (11/74)	11/15 (3/7)	2–15	NP	PVZ	Ref. 74
Mouse	UbC	GFP	NP	13 (19/146)	19/31 (19)[d]	1–12	NP	INFZ	Ref. 74
Mouse	CAG	GFP	NP	10% (3/29)	3/3 (3)[i]	N/D	NS	INF M	Ref. 105
Mouse	CAG	GFP	NP	25% (28/110)	15/23 (2)*	1–8	NP	INF T	Ref. 52
Mouse	CMV	GFP	NP	18% (7/40)	7/7 (5/6)*	N/D	4/7 (4)	INF T	Ref. 52
Mouse	Rho	GFP	NP	22% (13/60))	9/11 (1)*	N/D	NS	INF T	Ref. 52
Mouse	RG	GFP	NP	30% (18/60)	14/18 (1)*	N/D	NS	INF T	Ref. 52
Mouse	K12	GFP	NP	8% (5/60)	5/5 (2)*	N/D	NS	INF T	Ref. 52
Mouse	PGK	GFP	95% (187/197)	NP	1/2*	N/D	NP (37/40)	PVZ	Ref. 110
Mouse	PGK	GFP	93% (94/101)	NP	4/7 (0)	N/D	NS	PVZ + Pr	Ref. 110
Mouse	UbC	GFP	80% (163/203)	20% (32/163)	31/32 (31)	3–31	12/12	INVI Z	Ref. 141
Mouse	UbC	GFP	64% (97/151)	18% (37/97)	17/37 (17)	1–14	10/14	PVZ	Ref. 141
Mouse	CAG	GFP	61% (51/83)	34% (53/156)	NP/53	3–11	NP	INF T	Ref. 95
Mouse	CAG	GFP	100% (81/81)	43% (78/180)	NP	1–9	NP	INF B	Ref. 95
Mouse	H1	GFP shRNA	NP	NP	2/5	13–21	2/15 (2)	INF T	Ref. 128
Mouse	U6	CD8 shRNA	NP	NP	16/32 (8)*	2–6	NS	PVZ	Refs. 111,112
Mouse	U6	CD25 shRNA	NP	NP	11/42	N/D	NS	PVZ	Refs. 111,112
Mouse	U6	P53 shRNA	NP	NP	5/22	N/D	NS	PVZ	Refs. 111,112
Mouse	U6	Slc11a1 shRNA	NP	NP	3 (2)	N/D	NP	PVZ	Ref. 63
Mouse	U6	Ryk shRNA	NP	45% (18/40)	8/18 (8)	1–5	NP	PVZ	Ref. 75
Mouse	CMV	hGM-CSF	100% (95)	37% (35/95)	24/35 (24)	1–8	NS	PVZ	Ref. 107
Chicken	CMV	GFP	27% (20/73)[b]	N/A	1/1	1–5	20/44 (5)*	SGC	Ref. 80
Chicken	CMV	lacZ	27% (20/73)[b]	N/A	13/19	1–4	72/487 (13)*	SGC	Ref. 80
Chicken	PGK	GFP	4% (19/473)	N/A	3/6[c]	N/D	4/637 (4)	SGC	Ref. 15
Chicken	OVA	miR24	NP	N/A	1/NP	N/D	19/463 (7)[j]	SGC	Ref. 73
Chicken	OVA	hIFNβ1a	NP	N/A	1/NP	1	2/NP (1)[j]	SGC	Ref. 73
Chicken	EREOVA	hIFNβ1a	NP	N/A	3/NP	1	12/NP (3)[j]	SGC	Ref. 73
Quail	SYN	GFP	10% (8/80)	N/A	8/8 (2)[e]	1	19/143 (18)	SGC	Ref. 117
Porcine	PGK	GFP	NP	19% (46/244)	32/46 (30)	1–20	NS	PVZ	Ref. 48
Porcine	K14	GFP	NP	19% (16/86)	2/16 (2)	1–12	NS[h]	PVZ	Ref. 48
Porcine	CMV	GFP	82% (120/147)	33% (40/120)	37/40 (35)	1–5	NS	PVZ	Ref. 137
Porcine	PGK	GFP	NP	NP	3 (3)	1–2	14/26 (9)	PVZ	Ref. 47
Bovine	PGK	GFP	45±22% (227)	NS	NS	NS	NS	PVZ	Ref. 48
Bovine	PGK	GFP	40±17% (227)	NS	NS	NS	NS	PVO	Ref. 48
Bovine	PGK	GFP	58±7% (182)	NS	NS	NS	NS	PVZ	Ref. 30
Bovine	PGK	GFP	35±6% (248)	NS	NS	NS	NS	PVO	Ref. 30
Bovine	PGK	GFP	14±7% (126)	NS	NS	NS	NS	LM INF	Ref. 30
Bovine	UbC	GFP	30% (42/139)	NS	NS	NS	NS	PVO	Ref. 34
Bovine	PGK	GFP	25% (12/48)	50% (4/8)	4/4 (4)	4/12	1/1 (1)	PVO	Ref. 49
Bovine	PGK	GFP	22% (79/357)	22% (4/17)	0/4 (0)	NS	NS	PVO	Ref. 49

Continued

Table 2.—*Continued*

Species	Promoter	Transgene	%Embryo Viability (No. of Viable Treated Cells/Total Cells)	%Born (No. of Birth/ Transferred Embryos)	No. of F_0 Transgenic/ Total Examined (Transgene Expression)	F_0 Vector Copy No.	No. of F_1 Transgenic/Total Examined (Transgene Expression)	Method	Ref. No(s).
					Male cells				
Mice	CMV	LacZ	N/A	N/A	NP/44 (0)	N/D	NS	SSC (a, p)	Ref. 88
Mice	EF	LacZ	N/A	N/A	8/28 (8)[f]	N/D	NS	SSC (a)	Ref. 88
Mice	EF	LacZ	N/A	N/A	23/23 (23)[f]	N/D	NS	SSC (p)	Ref. 88
Rat	CMV	GFP	N/A	N/A	3/3 (3)[g]	1–2	13/44 (1)	SSC (p)	Ref. 41
Rat	EF	LacZ	N/A	N/A	42/730 (42)	1	5.8%	SSC (p)	Ref. 114

NS, studies were not performed to produce F_0 and/or F_1 progeny; NP, specific nos. were not provided in the study, but the experiments were performed; N/A, not applicable; N/D, not determined. *Methods*: SSC, transduced spermatogonial or germline stem cells injected into the seminiferous tubules in either adults (a) or pups (p); PV, direct injection into perivitelline space with and without back pressure (+Pr) into zygote (Z) or unfertilized oocytes (O); INF, infection into zona pellucida-denuded fertilized eggs at two-cell (T), blastocyst (B), or morulae stage (M); LM INF, infection into laser-microdrilled fertilized eggs; SGC, subgerminal cavity under the embryonic disk or unfertilized oocytes (O); INF, infection into zona pellucida-denuded fertilized eggs at two-cell (T), blastocyst (B), or morulae stage (M); LM INF, infection into laser-microdrilled fertilized eggs; SGC, subgerminal cavity under the embryonic disk; INVI, intracytoplasmic viral injection into zygotes (Z). *Promoters*: UbC, human ubiquitin C promoter; CMV, cytomegalovirus promoter; PGK, phosphoglycerokinase promoter; EF, elongation factor-1α promoter; CAG, chicken β-actin promoter with CMV enhancer; MH, myogenin promoter; SYN, human synapsin-1 promoter; K14, human keratin K14 promoter; RG, red pigment gene; Rho, rhodopsin; K12, keratin-12 gene; OVA, ovalbumin promoter and introns (2.8 kb); EREOVA, estrogen receptor element LCR cloned 5′ to the OVA (3.5 kb); H1, human H1 polIII promoter; U6, mouse U6 polIII promoter. *Transgenes*: hIFNβ1a, human interferon-β1a; miR24, ScFv-Fcmini-antibody; GFP, green fluorescent protein; lacZ, bacterial lacZ; hGM-CSF, human granulocyte macrophage colony stimulating factor; shRNA, short hairpin RNA molecule. [a]Outbred Sprague-Dawley rats had 53% and inbred Lewis rats had 33% transgenesis. [b]Did not provide the specific no. of embryos transduced with each distinct vector (lacZ and GFP). [c]Only 6 of 19 founders were examined, and 3 of the 6 examined were found to be transgenic. [d]Infection was performed using a range of multiplicities of infections (MOI) between 8 and 2,000. The low dose (MOI 8–80) only produced 1 transgenic mouse of 8 live births (12.5%), decreasing the overall efficiency using INF. [e]All 8 of the founders were transgenic, and only 2 of 8 founders were examined for GFP expression, of which both were positive. [f]SSC at 6 mo were found to colonize only using donor cells from pups (5/5 tested) but not from adults (0/4). [g]Personal communication with Dr. F. Kent Hamra (Univ. of Texas at Southwestern, Dallas, TX). [h]Four of eleven F_1 embryos at the 2–4 cell stage were transgenic. [i]Only 2 of 3 mice were shown (the 3rd mouse was assumed to be transgenic and GFP positive). [j]Gene expression was noted by Northern blot or ELISA in some of the animals *Not all animals were examined for transgene expression. Nos. in parentheses are the transgene-positive animals examined by immunofluorescence or Western blot.

1.45%) compared with other strains, such as crossed F1 C57Bl/6 hybrids to SJL or C3H (3–5%) (126). For this reason, the pronuclear approach is limited when attempting to isolate transgenic animal lines back-crossed onto specific genetic backgrounds, which are difficult to modify by this approach. This difficulty often leads to a lengthy breeding strategy to generate an acceptable expressing transgenic line. These latter issues as well as the need to obtain special training and equipment to perform the technically challenging pronuclear injections have led researchers to explore the use of viral systems as an alternative system to manipulate pluripotent cells to generate higher transgenic efficiencies.

Other than a proof-of-concept study using wild-type Moloney leukemia retroviruses demonstrating chromosomal integration following the pronuclear injection method (123), there has been little interest in the utilization of this method with replication-defective retroviral and lentiviral vectors because of the availability of simpler systems for viral transduction as discussed below.

Viral vector-mediated gene transfer. The present methods, using both retroviral and lentiviral vector-mediated gene transfer, have been to either *1*) incubate fertilized cells minus their zona pellucida or early embryos with the vector system or *2*) directly inject the vectors into the perivitelline space or cytoplasm of zygotic cells. Details regarding the efficiency of transgenic animal production using these viral vector systems as well as the ability to promote long-term and, possibly, organ-specific expression in the context of lentiviral integration are discussed below.

Retroviruses. Many of the pioneering retrovirus studies were performed by Jaenisch and colleagues in the 1970s and early 1980s (54–57). These investigators incubated zona pellucida-digested fertilized mouse eggs or early-stage embryos with wild-type MLV to demonstrate the proof of concept that retroviruses could readily integrate and pass through the germ lines allowing for their propagation into subsequent generations. However, the resulting progeny demonstrated genetic mosaicism and viral silencing (57). Nearly three decades have passed, and the development and use of replication-defective retroviral vectors have been tested in cows (14), pigs (11), and monkeys (13), but the problems related to transgene silencing have persisted even though transgenic efficiency is higher than for pronuclear injections with DNA. To circumvent the problems associated with the simple retrovirus vectors derived from MLV, more complex retroviral vectors, also known as lentiviral vectors, designed from various strains of immunodeficiency virus from humans and horses have been examined.

Lentiviruses. UBIQUITOUS CELLULAR AND VIRAL PROMOTERS. Lois et al. (74) and Pfeifer et al. (105) opened the door for the use of replication-defective lentiviral vectors for transgenic applications (see Table 2 for a list of studies using lentiviral transgenesis). Lois et al. performed a proof-of-concept study in mice and rats showing that the injection of VSV-G-pseudotyped lentiviral vectors into the perivitelline space of fertilized oocytes could significantly increase the production efficiency of transgenic mice. Pfeifer et al. (105) also demonstrated that lentiviral vectors can be used to efficiently manipulate zona-free embryos to produce founder transgenic mice. The embryo viability following lentiviral vector transduction has been shown to be very high (generally >70%) in all of the

species studied, including mice (74, 107, 110), rats (74), pigs (137), and cows (48).

Confirming studies in mice (52, 107) and rats (81, 131) reported similar transgenic efficiencies (68–73% in mice and 22–46% in rats). However, strain-specific differences in the transgenic efficiencies have been noted between inbred Lewis (33%) (131) and Dahl SS (22%) (81) vs. outbred Sprague-Dawley (53–59%) rats (74, 131). Subsequent breeding of the founder mice (52, 141) and rats (81, 131) resulted in 56–74% and 28–50%, respectively, of the F_1 population being transgenic with >93% of the F_1 mice and rats continuing to express green fluorescent protein (GFP) (52, 110, 131). To produce organ-specific expression following lentiviral transgenesis using ubiquitous promoters, Okada et al. (95) manipulated zona pellucida-denuded embryos at the blastocyst stage to generate transgenic animals that expressed GFP exclusively in the placenta. The manipulation of these germ cells at this stage in development was found to reduce the embryonic lethality associated with placental defects following delivery of the lentiviral vectors overexpressing Ets2, Mapk1, Mapk11, and Mapk14 (95). These studies showed that lentiviral vector administration at different stages in embryo development could affect the site of expression following mammalian differentiation even with the use of a promoter that is normally capable of ubiquitous activity.

Studies in larger animals, including pigs (48, 137) and cows (30, 34, 49), have also shown a very high level of efficiency of transgene integration in founder animals using lentiviral vectors. Pigs and cows have exhibited transgenic rates of 70–93% (48, 137) and 100% (49), respectively. A high rate of transgene expression was also observed in the founder pigs (64%) (47) and cows (100%) (49). Unlike the studies in mice, rats, and pigs using microinjection of the viral constructs in fertilized eggs, unfertilized bovine oocytes were used because of previous work by Hofmann et al. (48) demonstrating higher yields of GFP-expressing blastocysts after injection. In contrast, birds have been reported to exhibit a variable rate of transgenesis during founder production, from 4 to 27% (15, 73, 80, 117). The complexities of handling the embryos during subgerminal injection followed by the culturing of the manipulated embryos in a surrogate egg shell until hatching may play a role in the poor efficiencies in birds independently of the problems associated with transduction using lentiviral vectors. Even with the low rate of transgenesis in birds (Table 2), the germline transmission from the F_0 population was observed to propagate the integrated lentiviral vector into subsequent generations at rates up to 45% (15, 73, 80, 117).

As shown in Table 2, various cellular and viral promoters have been examined to assess transgene expression. Presently, five different ubiquitous promoters have been studied, including human ubiquitin C (UbC), cytomegalovirus (CMV), chicken β-actin with a CMV enhancer sequence (CAG), phosphoglycerokinase (PGK), and the elongation factor-1α (EF). The UbC and PGK promoters have been reported to drive the expression of reporter molecules in transgenic rats at >69% in F_0 rats (74, 131), mice (74, 141), pigs (47, 48), and cattle (49) as well as >92% in F_1 mice (110). Both the UbC and PGK promoters are generally considered to drive only weak transgene expression in the context of lentiviral vectors. More robust transgene expression can be achieved using the viral CMV and cellular CAG and EF promoters, and lentiviral vectors containing these promoters have been used to generate transgenic animals at high efficiency in F_0 pigs (>94%) (137) and mice (100%) (95, 107) and at 50% in F_1 mice (52).

These studies using the lentiviral vector containing ubiquitous promoter elements have shown an extremely efficient ability to modify early progenitor cells to produce transgenic animals with the capability to regulate gene expression on a persistent basis, in some cases for several generations. One species that could not generate transgenic founders using lentiviral vectors has been rhesus macaques (139). However, the reasons for this remain to be determined. It may be due to differences in the timing of the vector injection, or the vector sequences were not compatible with efficient integration into the rhesus macaque genome using a human lentiviral vector system (66). Further work is still needed to maximize transduction efficiency depending on the species being manipulated, and it may require the testing of alternative lentiviral vectors from different species, such as simian immunodeficiency virus (64, 89), to tailor specific strains for certain animals.

TISSUE-SPECIFIC PROMOTERS. The lentiviral vector system would have far greater utility if cell- and tissue-specific expression could be achieved in the transgenic animals. To address this issue, tissue-specific promoters were cloned into the lentiviral vectors to restrict transgene expression in the transgenic animals. Transgenic founders were produced at high efficiencies in mice (>70%) (52, 74) and rats (56%) (90), similar to the rates seen in transgenic animals generated using ubiquitous promoters. Lower efficiencies (13%) were found in F_0 pigs (48) using the basal keratinocyte-specific K14 promoter, but both of the founder transgenic pigs produced specific expression of GFP in various layers of the skin epithelium. In mice, Lois et al. (74) found that mice carrying the lentiviral integrants with myogenin promoter have an expression profile consistent with the published findings on the distribution of myogenin (16), specifically the skeletal muscles in the tongue, jaw, chest, and limbs but not the cardiac or smooth muscle, or other non-muscle-derived tissues. Similar tissue-specific expression was found in the thymus using the T lymphocyte proximal *lck* promoter (74).

Other studies have observed persistent neuron-specific expression of GFP using the synapsin-1 (SYN) promoter from F_0 and F_1 transgenic rats (90) and F_0 quails (117). However, there was ectopic expression of the transgene in the testes using the SYN promoter (90) as well as variable gene expression in different layers of the quail brain (117). In the study by Scott and Lois (117), silencing mechanisms could not be excluded as the reason for differential gene expression patterns in the neurons. Similar non-tissue-specific expression was noted by Ikawa et al. (52) in which retinal-specific expression of GFP was observed using red pigment (RG) and rhodopsin (Rho) promoters, but the corneal epithelial-specific promoter, K12, was found to have expression in the eye but also in other tissues, including the brain, skin, heart, and kidney.

Larger promoter (2.8–3.5 kb) regions from the ovalbumin (OVA) gene were used to study the expression of human IFNβ1a and miR24 (73) in chickens to determine whether *cis*-acting DNA elements could minimize the epigenetic effects. These transgene products were found to be exclusively expressed in the oviduct as determined by Northern blot analysis, but the level of expression was highly variable in the F_1 chickens, likely because of position effects of the proviral

integrant. This latter study shows that the insertion of additional *cis*-acting DNA elements, such as insulator sequences or locus control regions (LCR), may be required to restrict transgene expression when using tissue-specific promoters in lentiviral transgenesis as long as the vector genome size is not compromised, allowing for packaging and assembly during vector production.

RNA INTERFERENCE. Transgenic approaches have largely involved the overexpression of various transgenes, but the advent of RNA interference in mammals has allowed for the production of transgenic animals with the ability to decrease expression of specific genes (42, 115, 121). Recently, short hairpin RNA (shRNA) molecule overexpression has become popular using lentiviral transgenesis, with low-to-moderate transgenic efficiencies (13–53%) observed in founder mice and rats harboring shRNA constructs (21, 63, 75, 111, 112, 128). Tiscornia et al. (128) used transgenic GFP mice to demonstrate that shRNA targeted against the GFP gene would significantly reduce fluorescence intensity in double-transgenic (GFP^+/GFP $shRNA^+$) founder and progeny F_1 mice.

Because of the ease and efficiency of lentiviral transgenesis, more sophisticated studies using RNA interference (RNA_i) knockdown technology have been pursued. Rubinson et al. (111, 112) were able to generate 23–50% founder transgenic mice with various shRNA constructs in the lentiviral vector. In one group, transgenic mice overexpressing CD8 shRNA led to tremendous silencing of CD8 expression by 87–94% compared with control mice, but the loss in the number of mature CD8(+) cells (20–100%) did not directly correlate with the decreased level of gene expression (111, 112), likely because of a position effect of the proviral integrants or other epigenetic modifications. Subsequent studies by Kissler et al. (63) demonstrated that RNA_i silencing of Slc11a1, which downregulates Nramp1 protein, reduced the frequency of type 1 diabetes mellitus following the genetic modification of nonobese diabetic (NOD) zygotes. The NOD is a mouse strain that is notoriously difficult to manipulate using standard pronuclear injections. Lu et al. (75) discovered that Ryk was a novel biological receptor for Wnt in vivo by chronic knockdown of Ryk using shRNA expressed by lentiviral vectors. In this study, 38% of the mice were transgenic, but there was mosaicism in their genetic background, as determined by Southern blot analyses, similar to that found in F_0 transgenic rats overexpressing shRNA to *Dazl* (21). Thus further breeding was necessary to segregate the integrants to obtain progeny with only a single copy of the lentiviral vectors (75).

Overall, lentiviral vectors with various types of expression cassettes have demonstrated extreme flexibility to generate small and large transgenic animals with relatively high efficiencies. These studies have documented that the lentiviral vector system can regulate gene expression using over- and underexpression constructs, even in animal species and strains that were previously found to be relatively resistant to genetic manipulation using standard pronuclear DNA injection methods (63, 107). Large numbers of founder animals using lentiviral transgenesis can be generated without the need to generate individual transgenic lines in a time-consuming and laborious process. This would provide investigators the ability to expedite their phenotypic analyses of lentiviral vector-generated transgenic animals within a short (<7 wk) period of time (119). The importance of the lentiviral vector system has been clearly demonstrated by numerous investigators over the past few years, but several problems associated with this approach still need further study and will be discussed below (see ISSUES STILL TO CONSIDER REGARDING TRANSGENESIS USING LENTIVIRAL VECTORS).

Nuclear transfer using genetically modified embryonic or somatic donor cells. This approach involves the removal of the nucleus from mature oocytes and transfer of genetically manipulated embryonic or somatic cells under the zona pellucida. An electrical pulse is applied, resulting in the fusion of the two cell membranes and release of the donor nucleus into the oocyte. It is important to note that the donor embryonic or somatic cells are generally transfected with a plasmid DNA construct or transduced with an integrating viral vector system to express a selectable marker used to isolate a clonal population of transgenic cells. Since lentiviral vectors are extremely efficient in transducing cells in vitro, the amount of time needed to select for transgenic cells would be reduced, as shown by Golding et al. (34) in goats and Hofmann et al. (49) in cows. In both of these studies, however, only one transgenic birth was obtained, and it appears that the limiting step remains the physical transfer of the nucleus into recipient cells, which is 1–3% efficient in the generation of transgenic animals for most species (122).

Even though this method is about as efficient as pronuclear injection (94), there are a number of advantages in this approach vs. the classic pronuclear injections. First, the sex as well as the genetic background of the animal can be predetermined by choosing the appropriate genetic material. Second, this approach can bypass problems associated with mosaicism. Third, the donor embryonic stem or somatic cells can be manipulated in cell culture to control the level of specific gene expression and/or delete the function of a particular gene. Subsequently, these donor cells can be selected, propagated, and then stored for future transgenic applications. These benefits are offset by the major drawback associated with this approach, which is the major loss of embryos during pregnancy and a higher rate of neonatal deaths (29, 138). In the end, lentiviral vectors may only play a role in mediating the genetic modification of the donor nuclei before the injection and generation of transgenic animals.

Male-Derived Cells

In recent years, there has been an interest in investigating whether male cells, i.e., spermatozoa and spermatogonial stem cells, can be used to direct the transfer of exogenous DNA into female cells to generate transgenic animals. Alternative cells that have been genetically modified to produce transgenic animals from mice and other species are described below.

Sperm cell-mediated transgenesis. It has been found that exogenous DNA can be bound and taken up by sperm cells, allowing them to transfer their own as well as the internalized foreign DNA to unfertilized oocytes. The initial proof-of-concept study was performed by Brackett et al. (6), in which they transfected rabbit spermatozoa with SV40 DNA and used it to inseminate unfertilized ova in the female uterus. Nearly two decades later, Lavitrano et al. (70) extended this approach by producing transgenic mice using spermatozoa transfected with plasmid DNA. At that time, this was a major advance in the field, since this was more efficient (2- to 5-fold) than the

manipulation of female-derived cells. These findings were controversial (8), but there are now many studies demonstrating the production of transgenic animals from other species (120). Replacement of the live spermatozoa with membrane-damaged or "dead" unfertilized sperm cells could produce increased transgenic efficiencies (103). Other studies have shown that intracytoplasmic sperm injections of DNA, even with large DNA fragments between 0.25 and 0.5 Mb in YAC (85, 86), at the metaphase II stage can produce transgenic animals at high efficiencies (35%) compared with the conventional pronuclear microinjection method (<1% efficiency) (33).

In terms of viruses, high transgenic efficiency was observed by De Miguel and Donovan (22) using retroviral vectors in sperm, but it was difficult to determine whether their efficiency was any better than plasmid DNA, which was not compared. No studies have examined the potential effectiveness of the use of lentiviral vectors to modify sperm cells. Conceivably, if retroviral vectors prove to be superior to the use of naked DNA in sperm cells, then it is likely that lentiviral vectors would be even more effective and provide another step forward in transgenic efficiency using this approach. In the end, this method still requires refinement to isolate sperm cells as well as to optimize the conditions needed to maximize efficiency of the transduction of these particular cells using lentiviral vectors.

Spermatogonial or germline stem cells. An alternative method to manipulating spermatozoa has been to isolate and genetically modify spermatogonial stem cells (SSC) in vitro using plasmid DNA (58) and retroviral vectors (87). Subsequently, these cells are implanted into the seminiferous tubules of recipient male testes to produce transgenic spermatozoa. Studies by Nagano et al. (87) demonstrated that MLV-based vectors could transduce murine SSC, leading to the generation of transgenic offspring, of which only 4.5% of the pups inherited and expressed the marker gene.

To circumvent some of the problems associated with MLV vectors, recent studies have examined the use of lentiviral vectors as a vector to manipulate SSC from mice (88) and rats (41, 114). Nagano et al. (88) found that first-generation lentiviral vectors pseudotyped with VSV-G resulted in 30–50% higher transduction efficiency into mouse SSC compared with MLV-based retroviral vectors. The transplantation of the lentiviral vector-modified SSC derived from mouse pups resulted in the establishment of spermatogenic colonies in 100% of the recipient mouse testes. Unfortunately, the transmission of the lentiviral vector gene sequences was not assessed in subsequent mouse generations in this study. On the other hand, SSC from rats were manipulated by Hamra et al. (41), who found that 59% of the progeny were transgenic and derived from the cultured donor cells, and 50% (13 of 26) of those pups were transgenic with the lentiviral vector. In a more recent study, Ryu et al. (114) found lower transgenic efficiencies using lacZ (4.2%)- and GFP (6.3%)-expressing lentiviral vectors in the testes of recipient Sprague-Dawley rats than in the mouse studies. However, they observed that GFP was expressed in the subsequent progeny for at least three generations without silencing. It is important to note that the use of certain promoters following transduction into the SSC may play a pivotal role in determining whether a successful transgenic outcome is achieved. Transgenesis using the CMV promoter was not observed to induce expression of the reporter gene in mice (88). Similar results were seen in a study using the CMV promoter in rats [<8% of the animals expressed the transgene in F_1 rats (41)]. Swapping of the promoters to EF resulted in 100% (31 of 31) of the transgenic founder mice transplanted with the lentiviral vector-transduced SSC continuing to express β-galactosidase protein (88).

Overall, these are encouraging results compared with earlier experiences using MLV vectors (87) or pronuclear DNA injections (46), but the use of SSC appears far less efficient in male progenitor cells than with female germ cells. However, it remains an important and simple approach to develop transgenic animals having specific gene-targeted deletions, and improvements in manipulating and engrafting SSC into recipient testis are continuing to evolve.

ISSUES STILL TO CONSIDER REGARDING TRANSGENESIS USING LENTIVIRAL VECTORS

It is clear that lentiviral vectors have a strong future for transgenesis in various small to large animal species, but there are several lingering issues that require further investigation. Some of these problems cannot be solely attributed to the lentiviral vector system but to the field of transgenics in general.

Genetic Mosaicism: Effect of Lentiviral Integrant Copy Number

Several studies using mice (21, 51, 74), rats (131), and birds (117) have indicated that genetic mosaicism occurs using viral constructs, even though zygotes were used at the single cell stage. It is somewhat surprising to find mosaic founders with the use of fertilized single cell zygotes in mice (74) and rats (21, 131), but there are two potential scenarios that could lead to the creation of transgenic animals with a different genetic makeup: *1*) the lentiviral vector integration process is not complete before the initial cell division, and *2*) there may be prolonged persistence of the lentiviral preintegration complex.

To reduce the time frame for vector administration into the perivitelline space to nuclear translocation for genomic integration, the vector must undergo a complicated series of steps. The pseudotyped lentiviral vector must *1*) bind to its receptor and *2*) become internalized, *3*) uncoated, and *4*) reverse transcribed, and *5*) the preintegration complex must be translocated into the nucleus and *6*) integrated into the genome. Although wild-type lentiviruses take 1–2 h to integrate (44), VSV-G-pseudotyped lentiviral vectors have been shown to require at least 12 h for initial signs of integration, and most of the integration can take up to 48 h or longer (132). The elimination of several steps before the reverse transcription step could help to accelerate the integration following administration to the cell. To address this possibility, Yang et al. (141) produced an envelope-free lentiviral vector system for direct injection into the cytoplasmic space of the zygote, which would be able to circumvent many of the steps before the uncoating of the vector particle. This enabled these investigators to produce transgenic mice at an extremely high efficiency rate (97%) compared with the standard injection into the perivitelline space using the envelope-free (2.2%) or the VSV-G-pseudotyped lentiviral vector (46%). Southern blot analyses were not applied in this study, so it is not known whether this

approach resulted in less mosaicism. There was, however, a major drawback with this approach, since it had an adverse effect on the development of the modified embryos. These effects appeared to be due to the toxicity associated with the injection of a crude vector preparation rather than the invasiveness of the injection (141). Contaminating elements in the vector preparation could readily be eliminated by purifying the concentrated lentiviral vectors before injection (118, 129).

Another cause of genetic mosaicism could be the relatively long half-life of the lentiviral vector, which has been shown to be in the range of 8–9 h in vitro (144). There was an earlier study by Park et al. (100) that showed increased transduction into mouse hepatocytes following a partial hepatectomy 7 days after vector administration in vivo. This study suggests the possibility that lentiviral preintegration complexes could persist for several rounds or more of cell division before integration, resulting in genetic mosaicism. More information is needed to better understand the time frame of the survival of infectious lentiviral vector particles following introduction in the perivitelline space or cytoplasm of cell embryo to better control the factors contributing to variable integration into the transgenic animals. Since not all studies found this type of variable patterning, it would be interesting to know whether intrinsic vector-related sequences can be used to eliminate mosaicism by enhancing or destabilizing the preintegration complexes.

In general, the number of integrants found in lentiviral vector-produced transgenic animals has ranged between 1 and 31 copies in various studies. Unlike the concatemers found using plasmid DNA, only individual integration events have been reported using lentiviral vectors. The multiple proviral integrants observed in the founder animals have been segregated to produce transgenic mice (51) and rats (131) with only individual copies. Further studies of these animals have demonstrated that the individual copies of integrated lentiviral vectors are sufficient to elicit transgene expression and eliminate the variable gene expression found in the F_0 animals (131). The issue of genetic mosaicism remains a potential problem in transgenic animal production, and further investigations are needed to determine the factors mediating this process.

Variability of Transgene Expression: Role of Epigenetic Modifications

Regardless of the promoter-transgene combination, mosaic gene expression within distinct regions of specific organs or specific populations of cells in mice (63, 107, 124) and birds (80, 117) has been observed. Kissler et al. (63) found that GFP expression was cell type dependent, where 34% of the CD4(+) T cells were positive, whereas only 11% of B cells and 17.5% of granulocytes expressed GFP. Epigenetic silencing was associated with mosaic gene expression, since progeny F_1 mice demonstrated similar variegation. Using the CMV promoter, Punzon et al. (107) detected variable protein secretion of human granulocyte macrophage colony in transgenic mice that was independent of copy number and may have been the result of position effects of the integrant, leading to the activation of promoter silencing mechanisms. Complete and irreversible shutdown of GFP expression was observed in transgenic mice containing a modified *tet*-inducible promoter system, and this was believed to be induced by specific DNA sequences known as the Kruppel-associated box (KRAB) (124). In this study, the epigenetic silencing only occurred in the first few days of embryo development in the absence of the inducer, doxycycline, which allowed for the binding of the recombinant *tet*-repressor fusion protein, tTRKRAB, onto its target site in the promoter region. In the presence of doxycycline during this critical period, the silencing phenomenon could be avoided, and reversible expression of GFP could be achieved (124). This study definitively shows that *cis*-acting DNA elements can play a major role in epigenetic silencing of the promoters and are important factors to consider when generating transgenic animals.

In birds, expression of GFP was found in only 10% of quail neurons using the synapsin promoter (117), and variable transgene expression was detected in transgenic founders and progeny chickens by McGrew et al. (80). These examples appear to contradict the previous belief that lentiviral vector-mediated expression is more resistant to epigenetic silencing, such as methylation (83, 106) or histone deacetylation (96, 97), than when using MLV-based counterparts (57, 123) or pronuclear injection of plasmid DNA (17, 67). From these findings, it would appear that the DNA sequences found within the lentiviral vector constructs are responsible for the variegated expression and transgene silencing following integration.

For this reason, lentiviral vectors in recent studies were designed with specific deletions in the 3′-LTR (99, 146) in a process known as "self-inactivation " (or SIN) to eliminate the negative promoter interference between the LTR and the internal promoters. MLV-based vectors were methylated in the promoter region of the LTR by a variety of sophisticated cellular mechanisms, which evolved to eliminate the biological effects of genomic intruders (57, 123). However, it is clear that other vector-related sequences within either the remnant LTR or the expression cassette (promoter-transgene combination) can initiate the mobilization of silencing complexes to shut down gene expression from the proviral integrant, similar to the MLV vectors (25). Most of the transgenic F_0 animals studied to date have contained multiple integrants, which likely led to a desirable situation whereby some of the integrants were able to escape the silencing mechanisms and somewhat subvert the variability found in the transgene expression.

To address silencing issues in the context of transgenic animals, Hofmann et al. (47) bred progeny to segregate their proviral integrants and discovered that ~33% of the F_1 progeny were affected by hypermethylation of CpG dinucleotides within the human PGK promoter region, leading to low-level gene expression. An additional finding was that the site of proviral integration could markedly affect the level of GFP expression (47), and that the effects on transgene expression may be associated with a phenomenon known as position effect variegation, in which integration within particular chromosomal regions results in altered transgene expression (60). Although HIV has been shown to integrate a small percentage (2%) within 1 kb of CpG islands compared with MLV vectors (17%), this may be enough to provide the signals to promote vector silencing (140). It may be possible to overcome these negative *cis*-acting effects by the surrounding chromatin through the use of insulators or matrix/scaffold attachment regions (MAR/SAR) (26, 27, 45, 125), which can protect the expression cassettes from epigenetic shutdown. However, the

insertion of insulators or MAR/SAR into the lentiviral vector, usually into the 3′-LTR, may compromise vector titer and reduce the efficiency of transgenesis (see below).

Another factor involved in vector silencing could be the role of histone deacetylation, which was found to be an important mechanism in promoting silencing of lentiviral transgenes in mouse embryonic stem cells (76). Hofmann et al. (47) found that histone deacetylases, which were dependent on trichostatin A, did not play a major role in the maintenance of lentiviral vector silencing in their F_1 transgenic pigs. However, recent findings by Wang et al. (136) indicate that histone function may play a critical role in determining where the lentivirus integrates into the genome.

There are still many issues to consider regarding vector silencing with respect to the activation and recruitment of silencing machinery, which may be dependent on the inserted transgene as shown by Dalle et al. (20), who found that GFP, which has 60 CpG islands, potentiated the silencing of the β-globin gene. Elimination of the CpG islands by codon optimization in a GFP variant (dmGFP) was found to have markedly lower fluorescence intensity, by 2- to 14-fold, compared with wild-type GFP. However, none of the transduced cells in vitro or transgenic mice expressing the variant dmGFP was found to be silenced, unlike the integrants expressing the wild-type GFP. Most of the lentiviral transgenesis studies that have been performed to date have used GFP as the transgene marker, so much of the silencing found in these studies may need to be reexamined using codon-optimized transgenes without CpG islands or other recognition sites for silencing (20).

In the end, the selection of a specific internal promoter can be an important factor in the context of lentiviral transgenesis. Most of the cellular promoters discussed throughout this review have been able to maintain persistent transgene expression in either the founder or subsequent progeny for extended periods of time, although there were negative effects on silencing with the viral CMV promoter following transduction into either spermatogonial stem cells (41) in vitro or chicken eggs in vivo (80). The silencing mechanisms mediating the downregulation of the CMV promoter have not been fully elucidated, but the experimental results are consistent with previous gene therapy studies using this particular promoter in the liver (29, 36, 62), brain (79), and airway epithelia (142). Similar injection methods using the cellular PGK promoter in chickens (15) and cattle (49) were found to have ubiquitous expression in all tissues analyzed (15) with a constant level of GFP fluorescence (49). It is possible that the location of the proviral integration may have specific effects on particular promoter regions that would complicate the transgenesis approach using lentiviral vectors. There remains little doubt that further work on variable gene expression and silencing effects will be needed to validate the use of specific promoters, and that specific cellular promoters will likely prove to be superior over general viral promoters for lentiviral transgenesis.

Packaging Size

Empirically, early generation lentiviral vectors were able to produce functional vector particles even with a genome size of 16 kb (68). In reality, however, lentiviral vectors of this size may not be useful for biological applications, since the viral titers are dramatically reduced by up to 1,000-fold compared with lentiviral constructs of 5–7 kb. To calculate the cloning capacity, it is important to include the backbone of the lentiviral vector that is essential for the production of functional vector, which is generally ~1.6 kb (without WPRE) or ~2.2 kb (with the WPRE). From the previous studies by Kumar et al.(68), the maximal size of the lentiviral vectors was estimated to be ~13.5 kb.

As the vector genome sizes were increased to 7–9 kb, the vector titer was only reduced by 10–30% compared with the smaller vectors. In terms of transgenesis, bacterial lacZ-containing vectors (~6 kb), which are ~50% larger than GFP-containing vectors (~4 kb), were found to have an approximate twofold reduction in avian transgenesis (80), implicating a size-dependent effect on transgenic efficiency. However, rodent transgenesis using vector constructs similar to the lacZ vectors in the chicken study (80) had 45% transgenic efficiency in the founders, comparable (45%) with previous avian studies using smaller GFP-expressing lentiviral vectors with heterologous promoters. The differences in the transgenic efficiencies were likely attributable to differences in the vector stock titers (3-log orders between lacZ vs. GFP) or the use of an alternative non-primate-derived immunodeficiency viral vector system from horses (equine infectious anemia virus; EIAV) (80), in which the size of the expression cassette may have a greater effect on transgenesis compared with more widely used HIV-based vectors.

Expression cassettes (promoter-transgene) markedly larger than 4 kb would likely have reduced transgenesis rates using lentiviral vectors because of effects on vector integration, so studies are needed to determine the upper limit of the lentiviral vector packaging capacity. At the present time, however, the issue of packaging size may not be a major limitation, since promoter-transgene constructs <10 kb within the scientific community are fairly prevalent, so this approach should become a routine method in animal transgenesis.

Vector Toxicity

Early studies by Burns et al. (10) using VSV-G-pseudotyped retroviral vectors demonstrated fusogenic and other cell toxicity on cultured cells. It remains to be determined what optimal vector dose would promote efficient integration into pluripotent cells from different species; it may require the use of alternative viral glycoproteins that are less harmful to the cell (19, 59, 98, 108). The recent study by Yang et al. (141) demonstrating highly efficient transgenesis using an envelope-free lentiviral vector injected into the zygotic cytoplasm may circumvent some of the problems associated with the vector-related toxicity caused by the VSV-G envelope protein. However, further refinement of the technique to physically deliver the vector into the cytoplasm will be required to minimize the adverse effects on embryo development as noted by the authors using this approach (141).

Lentiviral Vector Integration and Its Implication for Gene Therapy

In light of recent gene therapy studies documenting leukomogenesis in young children treated with retroviral-manipulated hematopoietic stem cells (37, 38), the method of lentiviral transgenesis may have an alternate use as a preclinical model

to screen lentiviral vector constructs for problems related to insertional mutagenesis.

Previously, Montini et al. (84) demonstrated that MLV-based retroviral vectors activated a dose-dependent acceleration of tumor onset, and clinical trials using MLV-based retroviral vectors found integration to occur within the LMO2 region, resulting in leukomogenesis (37, 38). However, there may be several mitigating factors that preclude replication-defective lentiviral vectors as a potential prooncogenic vehicle. First, lentiviral vectors, even at high loads, did not exacerbate tumorigenesis in vitro (84), and there have been no observations of oncogenesis in lentiviral vector-treated animals in vivo. Second, high HIV-1 viral loads in humans have not been conclusively shown to promote tumorigenesis as a direct result of lentiviral integration (53). These findings appear to support the safety of lentiviral vectors derived from immunodeficiency viruses vs. their predecessor, Moloney leukemia retroviruses. As yet, there have been no reports of oncogenesis as a result of lentiviral transgenesis in different animal species. However, in utero and early neonatal administration of lentiviral vectors derived from horses was found to potentiate tumor formation in mouse livers and lungs in vivo, although this was not the case with lentiviral vectors derived from humans (127). This may suggest that the human lentiviral vectors are safer than those of other species, but this still may not be a true indicator of their nononcogenic potential, since MLV vectors were only found to be leukemogenic after transplanting transduced hematopoietic stem cells into human infants (37, 38). Presently, it remains to be determined how differences in lentiviral vector integration compared with the prooncogenic MLV can lead to the avoidance of insertional mutagenesis, even in the face of multiple integration events throughout the genome (Table 2).

It was initially believed that retroviral integration, including lentivirus, was completely random, but previous studies by Schroder et al. (116) in 2002 demonstrated integration of wild-type HIV-1 into actively transcribing genes and regional integration hotspots in which a small 2.4-kb area accounted for 1% of the integration sites using a cell culture system. This was subsequently validated by other groups (72, 82, 136, 140). Consistent with the wild-type HIV study, De Palma et al. (23) found that lentiviral vectors based on HIV-1 integrated within transcriptional units, but these investigators found that the integrated lentiviral vector had an attenuated ability to promote anomalous transcription because of the distal site of integration from the promoter region, unlike MLV, which had a 20% probability of inserting into a promoter region. Additional analyses of lentivirus integration by Wang et al. (136) demonstrated that HIV integration was strongly associated with several histone posttranslational modifications linked to active transcription (H3 acetylation, H4 acetylation, and H3 K4 methylation) and negatively associated with inhibitory modifications (H3 K27 trimethylation and DNA CpG methylation), repetitive elements (human endogenous retroviruses and LINE elements), and α-satellites.

In terms of transgenic animals, little is known as to whether lentiviral vector integration differs from cell culture systems, and whether any of these recent findings by Wang et al. (136) would be relevant. Recent work by Bryda et al. (9) and Michalkiewicz et al. (81) using a linker ligation-mediated PCR (LM-PCR) approach found that the integration pattern of lentiviral vectors was dispersed with no preference to any particular chromosome in transgenic rats. Bryda et al. noted that the EGFP-expressing parental rats containing multiple vector integrations resulted in the segregation of the integrated vector genome within F_1 progeny, leading to the loss of transgene expression depending on the site of chromosomal integration. Michalkiewicz et al. found that the integration pattern was largely within intervening sequences and/or noncoding regions in the genome. These results recapitulated the lentiviral vector integration patterns found in cell culture conditions (23, 69), with the caveat that there may be differences in the integration sites depending on the strain of immunodeficiency virus being used (39, 43). It is clear that more work is required to investigate the pattern and strength of transgene expression because of the location of the proviral integrant, and to determine whether segregation of these integrants in animals with multiple copies will affect the transgene expression.

Biosafety

Because of the enormous amount of work in the past decade on the biology of lentiviral vector systems, initial biosafety fears about the use and production of transgenic animals have been drastically reduced. It is clear, however, that unlike simple DNA constructs, viral vectors are more complex and could promote pathogenesis in humans, particularly those vectors predisposed to causing human disease, such as HIV. The split genome format of the lentiviral vector systems presently being employed in these types of studies are replication defective and, as such, treated as a level 2 biohazard. Although the precautions needed with this vector system are more involved than for the use of plasmid DNA, contaminating vector can be eliminated from most surfaces by simple cleaning of the affected areas with mild detergents and/or bleach followed by an ethanol wash. To date, there is an extremely low probability of generating replication-competent lentiviruses using the lentiviral vector system with three or more plasmids (28), which substantiates the safety of this system for users as well as animals.

These points should help to alleviate fears about using lentiviral vectors based on HIV as a method for transgenesis, especially for animals that will be subsequently handled by animal care and laboratory staff and used in a variety of animal and human studies. There is heightened public awareness regarding the consumption and biomedical use of genetically modified products, and as such, fear and resistance could have an adverse impact on biological products obtained from animals generated using lentiviral vectors. To alleviate biosafety concerns, clinical trials should elucidate the value of lentiviral vectors as a powerful and safe tool and address any potential problems, such as vector mobilization and the formation of replication-competent lentiviral vector (2, 51, 65, 71, 78). These studies as well as future research can demonstrate the complete safety of the lentiviral vectors for use as a method of animal transgenesis for biomedical studies and human consumption.

PERSPECTIVES

Lentiviral vectors have drawn significant interest from outside of the gene therapy field and have emerged as a promising new system for transgenic applications. It is becoming more

clear that the innate ability of lentiviral vectors to integrate into the host genome with high efficiency can be exploited by investigators to modify early pluripotent cells for transgenic animal production in various species, including those that have been experimentally difficult to manipulate using traditional pronuclear DNA injections, such as inbred strains of mice and rats as well as larger farm animals. The high efficiency in generating large numbers of founder transgenic animals, especially in mice and rats, would allow for more rapid screening of phenotypic changes without the need of laborious, time-consuming breeding protocols to isolate transgenic lines with defined integration sites following pronuclear injections. Even with the distinct advantages of using lentiviral vectors to create transgenic animals, several problems remain with regard to packaging limitations, transgene silencing, vector-related toxicity, and genetic mosaicism.

In consideration of the successes that have already been achieved using lentiviral vectors, it is clear that this system will continue to be improved to mitigate many of the present problems, and this system will emerge as an important technique for animal transgenesis. For now, the lentiviral vector system in the field of transgenics remains a highly promising application, and this method could revolutionize animal transgenesis by providing investigators with an alternative method to genetically modify animals at a more expeditious and cost-effective manner for biomedical as well as agricultural and pharmaceutical research.

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REFERENCES

1. **al-Shawi R, Kinnaird J, Burke J, Bishop JO.** Expression of a foreign gene in a line of transgenic mice is modulated by a chromosomal position effect. *Mol Cell Biol* 10: 1192–1198, 1990.
2. **Bank A, Dorazio R, Leboulch P.** A phase I/II clinical trial of beta-globin gene therapy for beta-thalassemia. *Ann NY Acad Sci* 1054: 308–316, 2005.
3. **Bishop JO.** Chromosomal insertion of foreign DNA. *Reprod Nutr Dev* 36: 607–618, 1996.
4. **Bishop JO, Smith P.** Mechanism of chromosomal integration of microinjected DNA. *Mol Biol Med* 6: 283–298, 1989.
5. **Bondioli KR, Biery KA, Hill KG, Jones KB, De Mayo FJ.** Production of transgenic cattle by pronuclear injection. *Biotechnology* 16: 265–273, 1991.
6. **Brackett BG, Baranska W, Sawicki W, Koprowski H.** Uptake of heterologous genome by mammalian spermatozoa and its transfer to ova through fertilization. *Proc Natl Acad Sci USA* 68: 353–357, 1971.
7. **Brinster RL, Chen HY, Trumbauer ME, Yagle MK, Palmiter RD.** Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc Natl Acad Sci USA* 82: 4438–4442, 1985.
8. **Brinster RL, Sandgren EP, Behringer RR, Palmiter RD.** No simple solution for making transgenic mice. *Cell* 59: 239–241, 1989.
9. **Bryda EC, Pearson M, Agca Y, Bauer BA.** Method for detection and identification of multiple chromosomal integration sites in transgenic animals created with lentivirus. *Biotechniques* 41: 715–719, 2006.
10. **Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK.** Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc Natl Acad Sci USA* 90: 8033–8037, 1993.
11. **Cabot RA, Kuhholzer B, Chan AW, Lai L, Park KW, Chong KY, Schatten G, Murphy CN, Abeydeera LR, Day BN, Prather RS.** Transgenic pigs produced using in vitro matured oocytes infected with a retroviral vector. *Anim Biotechnol* 12: 205–214, 2001.
12. **Chan AW.** Transgenic animals: current and alternative strategies. *Cloning* 1: 25–46, 1999.
13. **Chan AW, Chong KY, Martinovich C, Simerly C, Schatten G.** Transgenic monkeys produced by retroviral gene transfer into mature oocytes. *Science* 291: 309–312, 2001.
14. **Chan AW, Homan EJ, Ballou LU, Burns JC, Bremel RD.** Transgenic cattle produced by reverse-transcribed gene transfer in oocytes. *Proc Natl Acad Sci USA* 95: 14028–14033, 1998.
15. **Chapman SC, Lawson A, Macarthur WC, Wiese RJ, Loechel RH, Burgos-Trinidad M, Wakefield JK, Ramabhadran R, Mauch TJ, Schoenwolf GC.** Ubiquitous GFP expression in transgenic chickens using a lentiviral vector. *Development* 132: 935–940, 2005.
16. **Cheng TC, Hanley TA, Mudd J, Merlie JP, Olson EN.** Mapping of myogenin transcription during embryogenesis using transgenes linked to the myogenin control region. *J Cell Biol* 119: 1649–1656, 1992.
17. **Chevalier-Mariette C, Henry I, Montfort L, Capgras S, Forlani S, Muschler J, Nicolas JF.** CpG content affects gene silencing in mice: evidence from novel transgenes. *Genome Biol* 4: R53, 2003.
18. **Cockrell AS, Kafri T.** HIV-1 vectors: fulfillment of expectations, further advancements, and still a way to go. *Curr HIV Res* 1: 419–439, 2003.
19. **Cronin J, Zhang XY, Reiser J.** Altering the tropism of lentiviral vectors through pseudotyping. *Curr Gene Ther* 5: 387–398, 2005.
20. **Dalle B, Rubin JE, Alkan O, Sukonnik T, Pasceri P, Yao S, Pawliuk R, Leboulch P, Ellis J.** eGFP reporter genes silence LCRbeta-globin transgene expression via CpG dinucleotides. *Mol Ther* 11: 591–599, 2005.
21. **Dann CT, Alvarado AL, Hammer RE, Garbers DL.** Heritable and stable gene knockdown in rats. *Proc Natl Acad Sci USA* 103: 11246–11251, 2006.
22. **De Miguel MP, Donovan PJ.** Determinants of retroviral-mediated gene delivery to mouse spermatogonia. *Biol Reprod* 68: 860–866, 2003.
23. **De Palma M, Montini E, Santoni de Sio FR, Benedicenti F, Gentile A, Medico E, Naldini L.** Promoter trapping reveals significant differences in integration site selection between MLV and HIV vectors in primary hematopoietic cells. *Blood* 105: 2307–2315, 2005.
24. **Ebert KM, Selgrath JP, DiTullio P, Denman J, Smith TE, Memon MA, Schindler JE, Monastersky GM, Vitale JA, Gordon K.** Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression. *Biotechnology* 9: 835–838, 1991.
25. **Ellis J.** Silencing and variegation of gammaretrovirus and lentivirus vectors. *Hum Gene Ther* 16: 1241–1246, 2005.
26. **Emery DW, Yannaki E, Tubb J, Nishino T, Li Q, Stamatoyannopoulos G.** Development of virus vectors for gene therapy of beta chain hemoglobinopathies: flanking with a chromatin insulator reduces gamma-globin gene silencing in vivo. *Blood* 100: 2012–2019, 2002.
27. **Emery DW, Yannaki E, Tubb J, Stamatoyannopoulos G.** A chromatin insulator protects retrovirus vectors from chromosomal position effects. *Proc Natl Acad Sci USA* 97: 9150–9155, 2000.
28. **Escarpe P, Zayek N, Chin P, Borellini F, Zufferey R, Veres G, Kiermer V.** Development of a sensitive assay for detection of replication-competent recombinant lentivirus in large-scale HIV-based vector preparations. *Mol Ther* 8: 332–341, 2003.
29. **Everett RS, Evans HK, Hodges BL, Ding EY, Serra DM, Amalfitano A.** Strain-specific rate of shutdown of CMV enhancer activity in murine liver confirmed by use of persistent [E1(−), E2b(−)] adenoviral vectors. *Virology* 325: 96–105, 2004.
30. **Ewerling S, Hofmann A, Klose R, Weppert M, Brem G, Rink K, Pfeifer A, Wolf E.** Evaluation of laser-assisted lentiviral transgenesis in bovine. *Transgenic Res* 15: 447–454, 2006.
31. **Filipiak WE, Saunders TL.** Advances in transgenic rat production. *Transgenic Res* 15: 673–686, 2006.
32. **Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L.** Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat Genet* 25: 217–222, 2000.
33. **Giraldo P, Montoliu L.** Size matters: use of YACs, BACs and PACs in transgenic animals. *Transgenic Res* 10: 83–103, 2001.

34. **Golding MC, Long CR, Carmell MA, Hannon GJ, Westhusin ME.** Suppression of prion protein in livestock by RNA interference. *Proc Natl Acad Sci USA* 103: 5285–5290, 2006.
35. **Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH.** Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc Natl Acad Sci USA* 77: 7380–7384, 1980.
36. **Guo ZS, Wang LH, Eisensmith RC, Woo SL.** Evaluation of promoter strength for hepatic gene expression in vivo following adenovirus-mediated gene transfer. *Gene Ther* 3: 802–810, 1996.
37. **Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M, Fischer A.** A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 348: 255–256, 2003.
38. **Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JI, de Saint Basile G, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, Macintyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A, Cavazzana-Calvo M.** LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 302: 415–419, 2003.
39. **Hacker CV, Vink CA, Wardell TW, Lee S, Treasure P, Kingsman SM, Mitrophanous KA, Miskin JE.** The integration profile of EIAV-based vectors. *Mol Ther* 14: 536–545, 2006.
40. **Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL.** Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* 315: 680–683, 1985.
41. **Hamra FK, Gatlin J, Chapman KM, Grellhesl DM, Garcia JV, Hammer RE, Garbers DL.** Production of transgenic rats by lentiviral transduction of male germ-line stem cells. *Proc Natl Acad Sci USA* 99: 14931–14936, 2002.
42. **Hannon GJ.** RNA interference. *Nature* 418: 244–251, 2002.
43. **Hematti P, Hong BK, Ferguson C, Adler R, Hanawa H, Sellers S, Holt IE, Eckfeldt CE, Sharma Y, Schmidt M, von Kalle C, Persons DA, Billings EM, Verfaillie CM, Nienhuis AW, Wolfsberg TG, Dunbar CE, Calmels B.** Distinct genomic integration of MLV and SIV vectors in primate hematopoietic stem and progenitor cells. *PLoS Biol* 2: e423, 2004.
44. **Hewlett IK, Geyer SJ, Hawthorne CA, Ruta M, Epstein JS.** Kinetics of early HIV-1 gene expression in infected H9 cells assessed by PCR. *Oncogene* 6: 491–493, 1991.
45. **Hino S, Fan J, Taguwa S, Akasaka K, Matsuoka M.** Sea urchin insulator protects lentiviral vector from silencing by maintaining active chromatin structure. *Gene Ther* 11: 819–828, 2004.
46. **Hirabayashi M, Takahashi R, Ito K, Kashiwazaki N, Hirao M, Hirasawa K, Hochi S, Ueda M.** A comparative study on the integration of exogenous DNA into mouse, rat, rabbit, and pig genomes. *Exp Anim* 50: 125–131, 2001.
47. **Hofmann A, Kessler B, Ewerling S, Kabermann A, Brem G, Wolf E, Pfeifer A.** Epigenetic regulation of lentiviral transgene vectors in a large animal model. *Mol Ther* 13: 59–66, 2006.
48. **Hofmann A, Kessler B, Ewerling S, Weppert M, Vogg B, Ludwig H, Stojkovic M, Boelhauve M, Brem G, Wolf E, Pfeifer A.** Efficient transgenesis in farm animals by lentiviral vectors. *EMBO Rep* 4: 1054–1060, 2003.
49. **Hofmann A, Zakhartchenko V, Weppert M, Sebald H, Wenigerkind H, Brem G, Wolf E, Pfeifer A.** Generation of transgenic cattle by lentiviral gene transfer into oocytes. *Biol Reprod* 71: 405–409, 2004.
50. **Hollis RP, Stoll SM, Sclimenti CR, Lin J, Chen-Tsai Y, Calos MP.** Phage integrases for the construction and manipulation of transgenic mammals. *Reprod Biol Endocrinol* 1: 79, 2003.
51. **Humeau LM, Binder GK, Lu X, Slepushkin V, Merling R, Echeagaray P, Pereira M, Slepushkina T, Barnett S, Dropulic LK, Carroll R, Levine BL, June CH, Dropulic B.** Efficient lentiviral vector-mediated control of HIV-1 replication in CD4 lymphocytes from diverse HIV+ infected patients grouped according to CD4 count and viral load. *Mol Ther* 9: 902–913, 2004.
52. **Ikawa M, Tanaka N, Kao WW, Verma IM.** Generation of transgenic mice using lentiviral vectors: a novel preclinical assessment of lentiviral vectors for gene therapy. *Mol Ther* 8: 666–673, 2003.
53. **Jacoby J.** Interview with Dr. Verma. *Gene Ther* 12: 950–953, 2005.
54. **Jaenisch R.** Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus. *Proc Natl Acad Sci USA* 73: 1260–1264, 1976.
55. **Jaenisch R, Fan H, Croker B.** Infection of preimplantation mouse embryos and of newborn mice with leukemia virus: tissue distribution of viral DNA and RNA and leukemogenesis in the adult animal. *Proc Natl Acad Sci USA* 72: 4008–4012, 1975.
56. **Jaenisch R, Harbers K, Jahner D, Stewart C, Stuhlmann H.** DNA methylation, retroviruses, and embryogenesis. *J Cell Biochem* 20: 331–336, 1982.
57. **Jahner D, Jaenisch R.** Integration of Moloney leukaemia virus into the germ line of mice: correlation between site of integration and virus activation. *Nature* 287: 456–458, 1980.
58. **Kanatsu-Shinohara M, Toyokuni S, Shinohara T.** Genetic selection of mouse male germline stem cells in vitro: offspring from single stem cells. *Biol Reprod* 72: 236–240, 2005.
59. **Kang Y, Stein CS, Heth JA, Sinn PL, Penisten AK, Staber PD, Ratliff KL, Shen H, Barker CK, Martins I, Sharkey CM, Sanders DA, McCray PB Jr, Davidson BL.** In vivo gene transfer using a nonprimate lentiviral vector pseudotyped with Ross River Virus glycoproteins. *J Virol* 76: 9378–9388, 2002.
60. **Karpen GH.** Position-effect variegation and the new biology of heterochromatin. *Curr Opin Genet Dev* 4: 281–291, 1994.
61. **Kay MA, Glorioso JC, Naldini L.** Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med* 7: 33–40, 2001.
62. **Kay MA, Li Q, Liu TJ, Leland F, Toman C, Finegold M, Woo SL.** Hepatic gene therapy: persistent expression of human alpha 1-antitrypsin in mice after direct gene delivery in vivo. *Hum Gene Ther* 3: 641–647, 1992.
63. **Kissler S, Stern P, Takahashi K, Hunter K, Peterson LB, Wicker LS.** In vivo RNA interference demonstrates a role for Nramp1 in modifying susceptibility to type 1 diabetes. *Nat Genet* 38: 479–483, 2006.
64. **Kitagawa R, Miyachi S, Hanawa H, Takada M, Shimada T.** Differential characteristics of HIV-based versus SIV-based lentiviral vector systems: gene delivery to neurons and axonal transport of expressed gene. *Neurosci Res* 57: 550–558, 2007.
65. **Kohn DB.** Lentiviral vectors ready for prime-time. *Nat Biotechnol* 25: 65–66, 2007.
66. **Kootstra NA, Munk C, Tonnu N, Landau NR, Verma IM.** Abrogation of postentry restriction of HIV-1-based lentiviral vector transduction in simian cells. *Proc Natl Acad Sci USA* 100: 1298–1303, 2003.
67. **Kues WA, Schwinzer R, Wirth D, Verhoeyen E, Lemme E, Herrmann D, Barg-Kues B, Hauser H, Wonigeit K, Niemann H.** Epigenetic silencing and tissue independent expression of a novel tetracycline inducible system in double-transgenic pigs. *FASEB J* 20: 1200–1202, 2006.
68. **Kumar M, Keller B, Makalou N, Sutton RE.** Systematic determination of the packaging limit of lentiviral vectors. *Hum Gene Ther* 12: 1893–1905, 2001.
69. **Laufs S, Guenechea G, Gonzalez-Murillo A, Zsuzsanna Nagy K, Luz Lozano M, del Val C, Jonnakuty S, Hotz-Wagenblatt A, Jens Zeller W, Bueren JA, Fruehauf S.** Lentiviral vector integration sites in human NOD/SCID repopulating cells. *J Gene Med* 8: 1197–1207, 2006.
70. **Lavitrano M, Camaioni A, Fazio VM, Dolci S, Farace MG, Spadafora C.** Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. *Cell* 57: 717–723, 1989.
71. **Levine BL, Humeau LM, Boyer J, MacGregor RR, Rebello T, Lu X, Binder GK, Slepushkin V, Lemiale F, Mascola JR, Bushman FD, Dropulic B, June CH.** Gene transfer in humans using a conditionally replicating lentiviral vector. *Proc Natl Acad Sci USA* 103: 17372–17377, 2006.
72. **Lewinski MK, Bisgrove D, Shinn P, Chen H, Hoffmann C, Hannenhalli S, Verdin E, Berry CC, Ecker JR, Bushman FD.** Genome-wide analysis of chromosomal features repressing human immunodeficiency virus transcription. *J Virol* 79: 6610–6619, 2005.
73. **Lillico SG, Sherman A, McGrew MJ, Robertson CD, Smith J, Haslam C, Barnard P, Radcliffe PA, Mitrophanous KA, Elliot EA, Sang HM.** Oviduct-specific expression of two therapeutic proteins in transgenic hens. *Proc Natl Acad Sci USA* 104: 1771–1776, 2007.
74. **Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D.** Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 295: 868–872, 2002.

75. **Lu W, Yamamoto V, Ortega B, Baltimore D.** Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth. *Cell* 119: 97–108, 2004.
76. **Ma Y, Ramezani A, Lewis R, Hawley RG, Thomson JA.** High-level sustained transgene expression in human embryonic stem cells using lentiviral vectors. *Stem Cells* 21: 111–117, 2003.
77. **MacDonald RJ, Hammer RE, Swift GH, Davis BP, Brinster RL.** Transgenic progeny inherit tissue-specific expression of rat elastase I genes. *DNA* 5: 393–401, 1986.
78. **Manilla P, Rebello T, Afable C, Lu X, Slepushkin V, Humeau LM, Schonely K, Ni Y, Binder GK, Levine BL, MacGregor RR, June CH, Dropulic B.** Regulatory considerations for novel gene therapy products: a review of the process leading to the first clinical lentiviral vector. *Hum Gene Ther* 16: 17–25, 2005.
79. **McCown TJ, Xiao X, Li J, Breese GR, Samulski RJ.** Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector. *Brain Res* 713: 99–107, 1996.
80. **McGrew MJ, Sherman A, Ellard FM, Lillico SG, Gilhooley HJ, Kingsman AJ, Mitrophanous KA, Sang H.** Efficient production of germline transgenic chickens using lentiviral vectors. *EMBO Rep* 5: 728–733, 2004.
81. **Michalkiewicz M, Michalkiewicz T, Geurts AM, Roman RJ, Slocum GR, Singer O, Weihrauch D, Greene AS, Kaldunski ML, Verma IM, Jacob HJ, Cowley AW Jr.** Efficient transgenic rat production by a lentiviral vector. *Am J Physiol Heart Circ Physiol* 293: H881–H894, 2007.
82. **Mitchell RS, Beitzel BF, Schroder AR, Shinn P, Chen H, Berry CC, Ecker JR, Bushman FD.** Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol* 2: E234, 2004.
83. **Mohamedali A, Moreau-Gaudry F, Richard E, Xia P, Nolta J, Malik P.** Self-inactivating lentiviral vectors resist proviral methylation but do not confer position-independent expression in hematopoietic stem cells. *Mol Ther* 10: 249–259, 2004.
84. **Montini E, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C, Sergi Sergi L, Benedicenti F, Ambrosi A, Di Serio C, Doglioni C, von Kalle C, Naldini L.** Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol* 24: 687–696, 2006.
85. **Moreira PN, Giraldo P, Cozar P, Pozueta J, Jimenez A, Montoliu L, Gutierrez-Adan A.** Efficient generation of transgenic mice with intact yeast artificial chromosomes by intracytoplasmic sperm injection. *Biol Reprod* 71: 1943–1947, 2004.
86. **Moreira PN, Pozueta J, Giraldo P, Gutierrez-Adan A, Montoliu L.** Generation of yeast artificial chromosome transgenic mice by intracytoplasmic sperm injection. *Methods Mol Biol* 349: 151–161, 2006.
87. **Nagano M, Shinohara T, Avarbock MR, Brinster RL.** Retrovirus-mediated gene delivery into male germ line stem cells. *FEBS Lett* 475: 7–10, 2000.
88. **Nagano M, Watson DJ, Ryu BY, Wolfe JH, Brinster RL.** Lentiviral vector transduction of male germ line stem cells in mice. *FEBS Lett* 524: 111–115, 2002.
89. **Nagata M, Takahashi M, Muramatsu S, Ueda Y, Hanazono Y, Takeuchi K, Okada K, Suzuki Y, Kondo Y, Suemori M, Ikeda U, Nakano I, Kobayashi E, Hasegawa M, Ozawa K, Nakatsuji N, Shimada K.** Efficient gene transfer of a simian immuno-deficiency viral vector into cardiomyocytes derived from primate embryonic stem cells. *J Gene Med* 5: 921–928, 2003.
90. **Nakagawa T, Feliu-Mojer MI, Wulf P, Lois C, Sheng M, Hoogenraad CC.** Generation of lentiviral transgenic rats expressing glutamate receptor interacting protein 1 (GRIP1) in brain, spinal cord and testis. *J Neurosci Methods* 152: 1–9, 2006.
91. **Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D.** In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 272: 263–267, 1996.
92. **Naviaux RK, Verma IM.** Retroviral vectors for persistent expression in vivo. *Curr Opin Biotechnol* 3: 540–547, 1992.
93. **Ohashi K, Park F, Kay MA.** Role of hepatocyte direct hyperplasia in lentivirus-mediated liver transduction in vivo. *Hum Gene Ther* 13: 653–663, 2002.
94. **Ohashi K, Park F, Schwall R, Kay M.** Efficient gene transduction to cultured hepatocytes by HIV-1 derived lentiviral vector. *Transplant Proc* 34: 1431–1433, 2002.
95. **Okada Y, Ueshin Y, Isotani A, Saito-Fujita T, Nakashima H, Kimura K, Mizoguchi A, Oh-Hora M, Mori Y, Ogata M, Oshima RG, Okabe M, Ikawa M.** Complementation of placental defects and embryonic lethality by trophoblast-specific lentiviral gene transfer. *Nat Biotechnol* 25: 233–237, 2007.
96. **Pannell D, Ellis J.** Silencing of gene expression: implications for design of retrovirus vectors. *Rev Med Virol* 11: 205–217, 2001.
97. **Pannell D, Osborne CS, Yao S, Sukonnik T, Pasceri P, Karaiskakis A, Okano M, Li E, Lipshitz HD, Ellis J.** Retrovirus vector silencing is de novo methylase independent and marked by a repressive histone code. *EMBO J* 19: 5884–5894, 2000.
98. **Park F.** Correction of bleeding diathesis without liver toxicity using arenaviral-pseudotyped HIV-1-based vectors in hemophilia A mice. *Hum Gene Ther* 14: 1489–1494, 2003.
99. **Park F, Kay MA.** Modified HIV-1 based lentiviral vectors have an effect on viral transduction efficiency and gene expression in vitro and in vivo. *Mol Ther* 4: 164–173, 2001.
100. **Park F, Ohashi K, Chiu W, Naldini L, Kay MA.** Efficient lentiviral transduction of liver requires cell cycling in vivo. *Nat Genet* 24: 49–52, 2000.
101. **Park F, Ohashi K, Kay MA.** The effect of age on hepatic gene transfer with self-inactivating lentiviral vectors in vivo. *Mol Ther* 8: 314–323, 2003.
102. **Park F, Ohashi K, Kay MA.** Therapeutic levels of human factor VIII and IX using HIV-1-based lentiviral vectors in mouse liver. *Blood* 96: 1173–1176, 2000.
103. **Perry AC, Wakayama T, Kishikawa H, Kasai T, Okabe M, Toyoda Y, Yanagimachi R.** Mammalian transgenesis by intracytoplasmic sperm injection. *Science* 284: 1180–1183, 1999.
104. **Pfeifer A.** Lentiviral transgenesis. *Transgenic Res* 13: 513–522, 2004.
105. **Pfeifer A, Ikawa M, Dayn Y, Verma IM.** Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. *Proc Natl Acad Sci USA* 99: 2140–2145, 2002.
106. **Pion M, Jordan A, Biancotto A, Dequiedt F, Gondois-Rey F, Rondeau S, Vigne R, Hejnar J, Verdin E, Hirsch I.** Transcriptional suppression of in vitro-integrated human immunodeficiency virus type 1 does not correlate with proviral DNA methylation. *J Virol* 77: 4025–4032, 2003.
107. **Punzon I, Criado LM, Serrano A, Serrano F, Bernad A.** Highly efficient lentiviral-mediated human cytokine transgenesis on the NOD/scid background. *Blood* 103: 580–582, 2004.
108. **Qian Z, Haessler M, Lemos JA, Arsenault JR, Aguirre JE, Gilbert JR, Bowler RP, Park F.** Targeting vascular injury using Hantavirus-pseudotyped lentiviral vectors. *Mol Ther* 13: 694–704, 2006.
109. **Reiser J, Harmison G, Kluepfel-Stahl S, Brady RO, Karlsson S, Schubert M.** Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. *Proc Natl Acad Sci USA* 93: 15266–15271, 1996.
110. **Ritchie WA, Neil C, King T, Whitelaw CB.** Transgenic embryos and mice produced from low titre lentiviral vectors. *Transgenic Res* 16: 661–664, 2007.
111. **Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Rooney DL, Ihrig MM, McManus MT, Gertler FB, Scott ML, Van Parijs L.** A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* 33: 401–406, 2003.
112. **Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Zhang M, McManus MT, Gertler FB, Scott ML, Van Parijs L.** Corrigendum: A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* 39: 803, 2007.
113. **Rulicke T, Hubscher U.** Germ line transformation of mammals by pronuclear microinjection. *Exp Physiol* 85: 589–601, 2000.
114. **Ryu BY, Orwig KE, Oatley JM, Lin CC, Chang LJ, Avarbock MR, Brinster RL.** Efficient generation of transgenic rats through the male germline using lentiviral transduction and transplantation of spermatogonial stem cells. *J Androl* 28: 353–360, 2007.
115. **Sandy P, Ventura A, Jacks T.** Mammalian RNAi: a practical guide. *Biotechniques* 39: 215–224, 2005.
116. **Schroder AR, Shinn P, Chen H, Berry C, Ecker JR, Bushman F.** HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* 110: 521–529, 2002.
117. **Scott BB, Lois C.** Generation of tissue-specific transgenic birds with lentiviral vectors. *Proc Natl Acad Sci USA* 102: 16443–16447, 2005.
118. **Segura MM, Garnier A, Durocher Y, Coelho H, Kamen A.** Production of lentiviral vectors by large-scale transient transfection of suspen-

sion cultures and affinity chromatography purification. *Biotechnol Bioeng*. In press.

119. **Singer O, Tiscornia G, Ikawa M, Verma IM.** Rapid generation of knockdown transgenic mice by silencing lentiviral vectors. *Nat Protoc* 1: 286–292, 2006.
120. **Smith K, Spadafora C.** Sperm-mediated gene transfer: applications and implications. *Bioessays* 27: 551–562, 2005.
121. **Snove O Jr, Rossi JJ.** Expressing short hairpin RNAs in vivo. *Nat Methods* 3: 689–695, 2006.
122. **Solter D.** Mammalian cloning: advances and limitations. *Nat Rev Genet* 1: 199–207, 2000.
123. **Stewart C, Harbers K, Jahner D, Jaenisch R.** X chromosome-linked transmission and expression of retroviral genomes microinjected into mouse zygotes. *Science* 221: 760–762, 1983.
124. **Szulc J, Wiznerowicz M, Sauvain MO, Trono D, Aebischer P.** A versatile tool for conditional gene expression and knockdown. *Nat Methods* 3: 109–116, 2006.
125. **Tajima S, Shinohara K, Fukumoto M, Zaitsu R, Miyagawa J, Hino S, Fan J, Akasaka K, Matsuoka M.** Ars insulator identified in sea urchin possesses an activity to ensure the transgene expression in mouse cells. *J Biochem (Tokyo)* 139: 705–714, 2006.
126. **Tesson L, Cozzi J, Menoret S, Remy S, Usal C, Fraichard A, Anegon I.** Transgenic modifications of the rat genome. *Transgenic Res* 14: 531–546, 2005.
127. **Themis M, Waddington SN, Schmidt M, von Kalle C, Wang Y, Al-Allaf F, Gregory LG, Nivsarkar M, Themis M, Holder MV, Buckley SM, Dighe N, Ruthe AT, Mistry A, Bigger B, Rahim A, Nguyen TH, Trono D, Thrasher AJ, Coutelle C.** Oncogenesis following delivery of a nonprimate lentiviral gene therapy vector to fetal and neonatal mice. *Mol Ther* 12: 763–771, 2005.
128. **Tiscornia G, Singer O, Ikawa M, Verma IM.** A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc Natl Acad Sci USA* 100: 1844–1848, 2003.
129. **Tiscornia G, Singer O, Verma IM.** Production and purification of lentiviral vectors. *Nat Protoc* 1: 241–245, 2006.
130. **Trono D.** Lentiviral vectors: turning a deadly foe into a therapeutic agent. *Gene Ther* 7: 20–23, 2000.
131. **van den Brandt J, Wang D, Kwon SH, Heinkelein M, Reichardt HM.** Lentivirally generated eGFP-transgenic rats allow efficient cell tracking in vivo. *Genesis* 39: 94–99, 2004.
132. **Van Maele B, De Rijck J, De Clercq E, Debyser Z.** Impact of the central polypurine tract on the kinetics of human immunodeficiency virus type 1 vector transduction. *J Virol* 77: 4685–4694, 2003.
133. **Verma IM, Weitzman MD.** Gene therapy: twenty-first century medicine. *Annu Rev Biochem* 74: 711–738, 2005.
134. **Wall RJ.** Pronuclear microinjection. *Cloning Stem Cells* 3: 209–220, 2001.
135. **Wall RJ, Kerr DE, Bondioli KR.** Transgenic dairy cattle: genetic engineering on a large scale. *J Dairy Sci* 80: 2213–2224, 1997.
136. **Wang GP, Ciuffi A, Leipzig J, Berry CC, Bushman FD.** HIV integration site selection: analysis by massively parallel pyrosequencing reveals association with epigenetic modifications. *Genome Res* 17: 1186–1194, 2007.
137. **Whitelaw CB, Radcliffe PA, Ritchie WA, Carlisle A, Ellard FM, Pena RN, Rowe J, Clark AJ, King TJ, Mitrophanous KA.** Efficient generation of transgenic pigs using equine infectious anaemia virus (EIAV) derived vector. *FEBS Lett* 571: 233–236, 2004.
138. **Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH.** Viable offspring derived from fetal and adult mammalian cells. *Nature* 385: 810–813, 1997.
139. **Wolfgang MJ, Eisele SG, Browne MA, Schotzko ML, Garthwaite MA, Durning M, Ramezani A, Hawley RG, Thomson JA, Golos TG.** Rhesus monkey placental transgene expression after lentiviral gene transfer into preimplantation embryos. *Proc Natl Acad Sci USA* 98: 10728–10732, 2001.
140. **Wu X, Li Y, Crise B, Burgess SM.** Transcription start regions in the human genome are favored targets for MLV integration. *Science* 300: 1749–1751, 2003.
141. **Yang SH, Agca Y, Cheng PH, Yang JJ, Agca C, Chan AW.** Enhanced transgenesis by intracytoplasmic injection of envelope-free lentivirus. *Genesis* 45: 177–183, 2007.
142. **Zabner J, Wadsworth SC, Smith AE, Welsh MJ.** Adenovirus-mediated generation of cAMP-stimulated Cl^- transport in cystic fibrosis airway epithelia in vitro: effect of promoter and administration method. *Gene Ther* 3: 458–465, 1996.
143. **Zennou V, Petit C, Guetard D, Nerhbass U, Montagnier L, Charneau P.** HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* 101: 173–185, 2000.
144. **Zhang B, Metharom P, Jullie H, Ellem KA, Cleghorn G, West MJ, Wei MQ.** The significance of controlled conditions in lentiviral vector titration and in the use of multiplicity of infection (MOI) for predicting gene transfer events. *Genet Vaccines Ther* 2: 6, 2004.
145. **Zufferey R, Donello JE, Trono D, Hope TJ.** Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* 73: 2886–2892, 1999.
146. **Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, Trono D.** Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol* 72: 9873–9880, 1998.