TRANSGENIC TECHNOLOGIES FOR ANIMALS AS BIOREACTORS

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Contents

Summary

It was in the early 1980s that the first transgenic mouse was reported. Transgenic animals are useful for studying human diseases as well as for the manufacture of pharmaceutical proteins. With knowledge of the complete DNA sequence of the human genome, and that of other species as well, transgenic animal models will become increasingly important for evaluation of gene function in the near future. No doubt this will accelerate researches into mechanisms of human diseases and aid drug discovery. Furthermore, production of pharmaceutical proteins in the milk of transgenic dairy animals has been achieved and successfully scaled up in a handful leading biotech companies. However, transgenic efficiency in domestic mammals has remained low, and the underlying mechanism of transgene integration is elusive.

In recent years, technical advances have alleviated, at least to some extent, the low efficiency of animal transgenics. Some newly developed technologies (see *Methods in Gene Engineering*) such as retrovirus-mediated gene transfer, sperm utilized as DNA carriers through ICSI have provided opportunities for improvements in animal transgenics. Most notably, the combination of DNA somatic cell transfection technologies with those of mammalian cloning has overcome a major hurdle in producing large transgenic animals: the very high cost of producing transgenic farm animals with conventional pronuclear microinjection. Furthermore, these techniques can provide the options of performing other genetic alterations, such as gene knock-in and knock-out in large animals. Additionally, advances in the understanding of the regulation of gene expression in eukaryotic cells have provided more information for the design of reliable and highly expressed transgenes. Newly emerging technologies in

animal transgenics provide exciting opportunities for researchers further to comprehend gene function in both experimental and economically important domestic animals. An exciting new era of precise genetic manipulations is on the horizon.

1. Introduction

The regulation of gene function and expression has become one of the main themes in modern biological research (see *Methods in Gene Engineering*). Transgenic mice were first reported by Gordon in 1980, followed in 1982 with a report of a changed phenotype caused by the expression of transgene (see also *Genetic Engineering of Mammalian Cells*). The methodology using direct DNA microinjection was later successfully repeated in 1985, producing transgenic animals in three other species: rabbits, sheep, and pigs. Animal gene alteration through homologous recombination was first reported by Capecchi in 1989. By the year 2000, an estimated 5000 genes had been inactivated in mice.

It has been well documented that animal transgenic technologies have provided a valuable tool for studying gene function and genetic diseases in humans, and hence their application in the biomedical fields is essential. Some of the many applications in biomedical science include: (1) genetically altered animals as organ or tissue donors (xenotransplantation), and (2) manufacture of pharmaceutical or nutriceutical recombinant proteins (see also *Protein Engineering*) through various animal organs, including the mammary gland, seminal vesicle gland, kidney and bladder. Apart from basic research, transgenesis will also continue to provide a powerful tool to modify economically important traits in agriculturally important animals. In spite of the great progress that has been made in many transgenic animal technologies, there is still much room for improvement. In this review, we intend to summarize the state of the art of mammalian transgenesis for the creation of bioreactors, and to reveal current imperfections and discuss the future directions of research pertaining to the technologies of exogenous gene transfer and regulation of transgene expression in transgenic animals.

2. Methods for Producing Transgenic Animals as Bioreactors

The definition of a transgenic animal is one that has an exogenous DNA incorporated into its genome and is able to transmit this to its progeny. There are currently several methods used to produce transgenic animals, depending on the species.

2.1. Producing Transgenic Animals by Direct DNA Microinjection into Zygote Pronuclei

To incorporate into an animal's genome, exogenous DNA must pass through cell membranes to get access to chromatin in the nucleus. In metaphase, the cell's nuclear envelope disappears, so the foreign DNA only needs to get into cytoplasm to come into contact with chromosomes. However, at interphase, even though the foreign DNA enters the cytoplasm, the chance of integrating into chromatin is still slim because the nuclear envelope separates the nuclear contents from the cytoplasm. Furthermore, foreign DNA is more likely to be degraded in the cytoplasm. Naturally, foreign DNA could enter animal cells through various virus infections, but it is impossible for a DNA

fragment to pass through the cell's membrane boundary.

In 1980, Gordon and his coworkers first reported that exogenous genes could be introduced into the mouse genome by direct insertion into the nuclei of early embryos. In their report, a recombinant plasmid, composed of segments of herpes simplex virus and simian virus 40 viral DNA inserted into the bacterial plasmid pBR322, was microinjected into pronuclei of fertilized mouse oocytes; two of 78 mice born after one series of microinjections carried the transgene. This was followed by a report that the fusion gene of the mouse metallothionein-I gene with the structural gene for a rat growth hormone was expressed in transgenic mice produced by DNA microinjection into the pronuclei of fertilized mouse eggs. The microinjection technique was systematically improved by comparing the factors affecting transgenic efficiency in mice. Later, domestic animals were successfully produced using DNA microinjection.

To date, numerous animals across a broad spectrum of mammalian species have been produced using DNA microinjection, including rabbits, sheep, pigs, cattle, goats, and rats. However, due to its high cost, this method does not appear to be commercially viable for large species such as the bovine. It has been estimated that producing a transgenic cow can cost as much as US$546 000 when *in vivo* zygotes are microinjected. Apparently, most of the cost is related to surgery for recovering zygotes from superovulated donors. This problem could be alleviated by production of embryos using *in vitro* maturation and fertilization. The first transgenic bull harboring the gene for human lactoferrin was reported in 1991, and was produced by microinjection of DNA into pronuclei of zygotes derived from *in vitro* maturation and fertilization of slaughterhouse-sourced oocytes. Producing transgenic animals through microinjection of *in vitro* derived zygotes has been applied to the large-scale transgenic production of cattle and goats in recent years.

The microinjection technique is perhaps the most straightforward approach to introducing DNA into the nuclei. Due to the ability of the cell membrane to heal, a localized rupture of the oolemma usually can recover immediately following microinjection. A finely drawn glass needle (tip size about 1 μm), loaded with DNA, is used for microinjection. Under a microscope, the micropipette is inserted into the ooplasm and penetrates the oolemma and nuclear envelope. Typically, about 2 pl of DNA solution (about 300 DNA molecules) is injected into the male pronucleus in mice. Surviving zygotes are transferred into synchronized pseudopregnant recipients to develop to term. Transgenic offspring can be identified with PCR screening (see also *Chemical Methods Applied to Biotechnology*), and further confirmation can be ensured by southern blotting and FISH (fluorescence *in situ* hybridization) analyses.

The microinjection procedure used in mice could be adapted for other animals; however, this presents many challenges. Unlike mice, most of the zygotes from domestic animals have a higher quantity of cytoplasmic lipids, which cause zygotes to appear opaque under the microscope. Centrifugation at 10 000 g to 15 000 g is necessary to stratify the opaque lipids in the cytoplasm in order to visualize the pronuclei in the zygotes of goats, cattle, and pigs. Moreover, the zygotic nuclear envelope of goats, cattle, and pigs at early pronuclear stage is less elastic than that of mice; therefore, microinjection often results in the nuclear membrane rupturing and/or DNA solution leaking into the

cytoplasm. To make transgenic domestic animals with an acceptable transgenic rate (5-10% of offspring born), the DNA concentration, the amount of solution delivered into the pronuclei and mechanical damage to the pronuclei or chromatin, and other factors need to be carefully considered. In addition, embryo culture systems and the embryo stages prior to their transfer should be optimized, since *in vitro* and *in vivo* environments may impose negative selection pressure on the survival of transgenic embryos. Nienmann and his colleagues reported that 16–20% of the piglets were transgenic following the transfer of 20–30 microinjected zygotes per recipient, compared with 8% transgenics when more than 30 zygotes were transferred per recipient in pigs, which suggests that *in vivo* conditions favor the development of non-transgenic embryos.

The rates at which foreign DNA integrates into an animal's genome (transgenic rate) varies across different mammalian species. Under optimal conditions, about 30% of mice offspring are transgenic, but the transgenic rate of offspring is much lower in domestic animals: 12–21% in rabbits, 5–10% in goats, 8.3% in sheep, 9.2% in pigs and 7–8% in the bovine. The reasons why the transgenic rate in domestic animals is lower remain to be determined. Wall pointed out that timing of microinjection could contribute to differences in integration efficiency. One obvious discrepancy is that DNA microinjection is usually performed at the zygotic G1 or early S phase in the mouse, but at the later S or G2 phase in domestic animals. Some evidence suggests that integration of foreign DNA occurs during the DNA replication in host cells, so it seems less likely that DNA integration can occur during the first cell cycle of a zygote in domestic animals. An attempt to microinject synchronized bovine zygotes at early, mid and later S phase was reported, but the results were not conclusive. It was unclear whether injection timing could affect DNA integration because of interference by the transient expression of non-integrated genes in the preimplantational embryos.

Other factors also need to be considered. When compared with mice the embryo viability of domestic animals dramatically decreases following micromanipulation, presumably because the *in vitro* culture system for embryos of domestic animals has not yet been optimized. The machinery in embryos used to repair DNA might also be involved in the integration of foreign DNA. A less than optimal culture system could certainly hinder the embryo's ability to repair its DNA, and in turn reduce its ability to integrate foreign DNA. Lastly, several variables such as the DNA dilution buffer and the concentration at which it is microinjected have not been optimized for domestic animals. To date, there have been no reports on the effect of DNA buffer composition, such as EDTA concentration, on the integration of DNA into the genome of domestic animals, although this has been shown to be very critical in mice. It has been demonstrated that EDTA could induce the chromosome-breaking activity. DNA double strand breaks could be exacerbated by microinjection of DNA solution containing EDTA; these breaks might provide the sites for DNA integration through ligation reactions. Nevertheless, it is practically impossible, due to the enormous amounts of labor and finance required, to do statistically adequate testing in large domestic animals.

2.2. Characteristics of Transgene Integration and the Mechanism of Mosaicism

The exogenous DNA delivered into pronuclei will persist in embryos either as genomic

integration or in episomal forms; otherwise it will face degradation in the cytosol as the embryo develops. Foreign DNA molecules introduced into the nucleus first align themselves in arrays of concatemers (mostly head to tail) through homologous recombination or random end-to-end joining, then these short or long concatemers enter into a site on one of the chromosomes. Early transgenic mouse studies suggested that integration often occurred at only one site on one chromosome; more data in recent years, however, showed that DNA integration could take place in multiple locations on either the same or different chromosomes depending on the sequences of DNA injected. DNA head to tail concatemers can quickly form in a few minutes after DNA is microinjected into mouse pronuclei. These concatemers could be detected in the embryos from one cell to blastocyst stage, and moreover, the exogenous DNA in blastomeres was distributed in a mosaic pattern. Whitelaw and his coworkers reported that as many as 62% of pronuclear microinjection-derived transgenic founders are the mosaics of transgenic and non-transgenic cells, which suggests that the majority of DNA injected into fertilized mouse eggs integrates after the first round of chromosomal replication.

It has been postulated that DNA repair mechanisms mediate transgene integration. Two DNA repair pathways, homologous recombination and nonhomologous recombination, are involved in the insertion of transgenes. Homologous recombination can promote transgene integration when the transgenes possess the same sequences as the endogenous genes. When transgenes do not share extensive genomic identity, integration might be the result of homologous matches of short sequences between transgenes and genomic sequences. The frequency of transgene integration through homologous recombination is very low in mammalian cells. Recent evidence clearly suggests that a pathway exists in the DNA repair mechanism of mammalian cells for the joining of nonhomologous ends, and is the preferred mechanism for the repair of double strand break (DSB) during mitosis in vertebrates. DNA repair through nonhomologous end-joining is believed to mediate the illegitimate insertion of exogenous DNA into chromatin.

DNA repair activity is regulated through cell-cycle stages. In G1 and early S phases, DNA DSB repair is characterized by nonhomologous recombinant repair in mammalian cells. Two discrete complexes are implicated in this pathway: the DNA-dependent protein kinase (DNA-PK) complex and the RAD 50 complex. However, DNA DSB repair through homologous recombination dominates in the S and G2 phases. RAD 52 complex and BRCA ½ complex mediate this pathway. Since the homologous integration of exogenous DNA in mammals is a very rare event, transgene integration probably occurs through nonhomologous recombination. As described above, due to the difficulty of injecting a smaller pronucleus at G1 phase, DNA microinjection usually takes place during S and G2 phases, and consequently exogenous DNA has very little chance of integrating through nonhomologous recombination during the first cell cycle. Therefore, we could assume that the transgene remaining episomally in the zygotes following its first cleavage would be most likely to integrate during the G1 and/or early S phase of the 2 cell or later stage embryo. However, the integration rate is constrained by the frequency of spontaneously occurring double strand breaks in the chromatin. When DNA integration occurs after the first cleavage, it results in transgenic mosaicism.

Glossary

Animal cloning:	The production of an animal with nuclear DNA identical to another animal.
Cell cycle:	Most eukaryotic cell cycles can commonly be divided into four phases: G1 before DNA synthesis occurs; S when DNA replication occurs; G2 after DNA synthesis; and M (Metaphase) when cell division occurs, yielding two daughter cells. A non-dividing state is called "quiescence" (G0).
Chromatin:	Complex of DNA, histones, and nonhistone proteins present in the nucleus of eukaryotic cells.
Germ line:	The lineage of cells that connect the generations. The sperm and the egg are examples of germ line cells.
Homologous recombination:	Genetic recombination involving exchange of homologous loci.
Nuclear transfer:	A general term for the process of cloning where the genetic information from a body cell is transferred to an egg cell whose DNA is removed.
Oolemma:	The cell membrane of an oocyte.
Protein kinase:	Enzyme catalyzing transfer of phosphate from ATP to hydroxyl side chains on proteins, causing changes in function.
Promoter:	DNA sequence that determines the site of transcription initiation for a RNA polymerase.
Reverse transcriptase:	RNA-directed DNA polymerase.
Somatic cell:	A body cell, as opposed to a germ line line cell.
Transgenesis:	The stable introduction of modified genes or genes from another animal or species into an animal's genome.
GFP:	Green fluorescent protein.
ICSI:	Intracytoplasmic sperm injection.
MII:	Metaphase II.
PCR:	Polymerase chain reaction.

Bibliography

Bachiller D., Schellander K., Peli J., and Ruther U. (1991). Liposome-mediated DNA uptake by sperm cells. *Molecular Reproduction and Development* **30**, 194–200. [This paper reports a method of gene transfer into spermatozoa by liposome transfection.]

Baguisi A., Behboodi E., Melican D.T., Pollock J.S., Destrempes M.M., Cammuso C., Williams J.L.,

Nims S.D., Porter C.A., Midura P., Palacios M.J., Ayres S.L., Denniston R.S., Hayes M.L., Ziomek C.A., Meade H.M., Godke R.A,. Gavin W.G., Overstrom E.W., and Echelard Y. (1999). Production of goats by somatic cell nuclear transfer. *Nature Biotechnology* **17,** 456–461. [The cloned goats were produced by somatic cell nuclear transfer using *in vivo* matured oocytes.]

Baldassarre H., Bilodeau A., Leduc M., Kafidi N., Gauthier M., Neveu N., Keefer C.L., Huang Y., Duguary F., Wang B., Zhou J.F., Lazaris A., and Karatzas C.N. (2000). Inheritance of a transgene and expression in transgenic BELE goat. *14th International Congress on Animal Reproduction.* Stockholm, July 2–6, 2000. Vol. 2, p. 251. [This paper reports the generation of transgenic goats producing a high level of hTPA in their milk.]

Baranov V.S., Brobohaty B., Tsymbalenko N.V., Tadlicek L., Gorbunova V.N., Kazakova T.B., Kovac L., and Bodja K. (1990). The possibility of the incorporation of macromolecules, including exogenous DNA, into the germ cells of male mice: the liposome method and Ca-P coprecipitation method. *Tsilogiia Genetikia* **24**, 52–55. [The methods of delivering protein into spermatozoa are described.]

Bawden W.S., Passey R.J., and Mackinlay A.G. (1994). The genes encoding the major milk-specific proteins and their use in transgenic studies and protein engineering. *Biotechnology and Genetic Engineering Review* **12**, 89–137. [The gene function and structures of the milk proteins are reviewed.]

Bishop J.O. and Smith P. (1989). Mechanism of chromosomal integration of microinjected DNA. *Molecular Biology and Medicine* **6,** 283–298. [The mechanisms of foreign gene integration in cultured cells and transgenic mice are proposed in this review.]

Bleck G.T., White B.R., Miller D.J., and Wheeler M.B. (1998). Production of bovine alpha-lactalbumin in the milk of transgenic pigs. *Journal of Animal Science* **76**, 3072–3078. [This describes how transgenic pigs were produced through microinjection of the bovine alpha-lactalbumin gene.]

Bowen R.A., Reed M., Schnieke A., Seidel G.E. Jr., Brink Z., and Stacey A. (1993). Production of transgenic cattle from PCR-screened embryos. *Theriogenelogy* **39,** 194. [This paper provides a method to identify transgenic embryos using PCR.]

Brackett B.G., Baranska W., Sawicki W., and Koprowski H. (1971). Uptake of heterologous genome by mammalian spermatozoa and its transfer to ova through fertilization. *Proceedings of the National Academy of Science USA* **68,** 353–357. [The first evidence of DNA transferring into spermatozoa is presented in this paper.]

Brem G., Hartl P., Besenfelder U., Wolf E., Zinovieva N., and Pfaller R. (1994). Expression of synthetic cDNA sequences encoding human insulin-like growth factor-1 (IGF-1) in the mammary gland of transgenic rabbits. *Gene* **149**, 351–355. [This paper reports an expression system where foreign proteins are synthesized specifically in the mammary gland of transgenic rabbits and secreted into the milk.]

Brinster R.L., Chen H.Y., Trumbauer M.E., Yagle M.K., and Palmiter R.D. (1985). Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proceedings of the National Academy of Science USA* **82**, 4438–4442. [Some of the important parameters that influence successful integration of foreign DNA into mouse chromosomes are described.]

Buhler T.A., Bruyere T., Went D.F., Stranzinger G., and Burki K. (1990). Rabbit beta-casein promoter directs secretion of human interleukin-2 into the milk of transgenic rabbits. *Biotechnology (NY)* **8,** 140–143. [This paper reports that transgenic rabbits may represent a convenient and economic system for the rapid production of biologically active protein in milk.]

Bulger M. and Groudine M. (1999). Looping versus linking: toward a model for long-distance gene activation. *Genes and Development* **13,** 2465–2477. [This review discusses the regulation of gene activation through long distant interaction between the regulatory elements.]

Burdon T.G. and Wall R.J. (1992). Fate of microinjected genes in preimplantation mouse embryos. *Molecular Reproduction and Development* **33,** 436–442. [This paper investigates the degradation and integration of the transgene following microinjection into mouse embryos.]

Capecchi M.R. (1989). Altering the genome by homologous recombination. *Science* **244,** 1288–1292. [This paper reports the transgenic animals produced through DNA homologous recombination.]

Cappello F., Stassi G., Lazzereschi D., Renzi L., Di Stefano C., Marfe G., Giancotti P., Wang H.J., Stoppacciaro A., Forni M., Bacci M.L., Turchi V., Sinibaldi P., Rossi M., Bruzzone P., Pretagostini R.,

Della Casa G., Cortesini R., Frati L., and Lavitrano M. (2000). hDAF expression in hearts of transgenic pigs obtained by sperm-mediated gene transfer. *Transplantation Proceedings* **32**, 895–896. [This paper reports transgenic pigs produced using spermatozoa as DNA carriers.]

Chan A.W., Chong K.Y., Martinovich C., Simerly C., and Schatten G. (2001). Transgenic monkeys produced by retroviral gene transfer into mature oocytes. *Science* **291,** 309–312. [This paper reports that transgenic rhesus monkeys carrying the green fluorescent protein (GFP) gene were produced by injecting pseudotyped replication-defective retroviral vector into the perivitelline space of the mature rhesus oocytes.]

Chan A.W., Homan E.J., Ballou L.U., Burns J.C., and Bremel R.D. (1998). Transgenic cattle produced by reverse-transcribed gene transfer in oocytes. *Proceedings of the National Academy of Science USA* **95,** 14028–14033. [This presents the results that reverse-transcribed gene transfer can take place in an oocyte in MII arrest of meiosis, leading to production of offspring, the majority of which are transgenic.]

Chang K., Qian J., Jiang M., Liu Y.H., Wu M.C., Chen C.D., Lai C.K., Lo H.L., Hsiao C.T., Brown L., Bolen J., Jr., Huang H.I., Ho P.Y., Shih P.Y., Yao C.W., Lin W.J., Chen C.H., Wu F.Y., Lin Y.J., Xu J., and Wang K. (2002). Effective generation of transgenic pigs and mice by linker based sperm-mediated gene transfer. *BMC Biotechnology* **2,** 5. [This paper reports a linker based sperm-mediated gene transfer method (LB-SMGT) that greatly improves the production efficiency of large transgenic animals.]

Chesne P., Adenot P.G., Viglietta C., Baratte M., Boulanger L., and Renard J.P. (2002). Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nature Biotechnology* **20**, 366–369. [This paper reports on the first cloned rabbits produced by somatic cell's nuclear transfer.]

Chung J.H., Whiteley M., and Felsenfeld G. (1993). A 5' element of the chicken beta-globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. *Cell* **73,** 505–514. [This paper provides the evidence that chicken beta-globin can function as an insulator.]

Cibelli J.B., Stice S.L., Golueke P.J., Kane J.J., Jerry J., Blackwell C., Ponce de Leon F.A., and Robl J.M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280,** 1256–1258. [Cloned cattle were produced by nuclear transfer using actively dividing somatic cells.]

Colman A. (1996). Production of proteins in the milk of transgenic livestock: problems, solutions, and successes. *America Journal of Clinical Nutrition* **63,** 639S–645S. [This review discusses the application of transgenic animals in the manufacture of pharmaceuticals.]

Coulibaly S., Besenfelder U., Miller I., Zinovieva N., Lassnig C., Kotler T., Jameson J.L., Gemeiner M., Muller M., and Brem G. (2002). Expression and characterization of functional recombinant bovine follicle-stimulating hormone (boFSHalpha/beta) produced in the milk of transgenic rabbits. *Molecular Reproduction and Development* **63,** 300–308. [This paper reports the generation of transgenic rabbits used for the bioreactor.]

Cousens C., Carver A.S., Wilmut I., Colman A., Garner I., and O'Neill G.T. (1994). Use of PCR-based methods for selection of integrated transgenes in preimplantation embryos. *Molecular Reproduction and Development* **39**, 384–391. [This study examines the fate of the ovine beta-lactoglobulin-human alpha 1-antitrypsin (AATB) minigene construct or a subfragment of this following microinjection into one-cell mouse embryos.]

Craigie R., Fujiwara T., and Bushman F. (1990). The IN protein of Moloney murine leukemia virus processes the viral DNA ends and accomplishes their integration *in vitro*. *Cell* **62,** 829–837. [This paper provides the evidence that the IN protein of Moloney murine leukemia virus (MoMLV) is the only viral protein required to accomplish the infection *in vitro*.]

Cummings W.J. and Zolan M.E. (1998). Functions of DNA repair genes during meiosis. *Current Topics in Developmental Biology* **37,** 117–140. [This article reviews the different aspects of DNA repair during meiosis.]

Dyck M.K., Gagne D., Ouellet M., Senechal J.F., Belanger E., Lacroix D., Sirard M.A., and Pothier F. (1999). Seminal vesicle production and secretion of growth hormone into seminal fluid. *Nature Biotechnology* **17,** 1087–1090. [This paper demonstrates that the mouse P12 gene promoter specific to the male accessory sex gland can be used to generate transgenic mice that express human growth hormone (hGH) in their seminal vesicle epithelium.]

Ebert K.M., Selgrath J.P., DiTullio P., Denman J., Smith T.E., Memon M.A., Schindler J.E., Monastersky G.M., Vitale J.A., and Gordon K. (1991). Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression. *Biotechnology (NY)* **9**, 835–838. [This paper reports the generation of transgenic goats producing hTPA in their milk.]

Ellis J., Tan-Un K.C., Harper A., Michalovich D., Yannoutsos N., Philipsen S., and Grosveld F. (1996). A dominant chromatin-opening activity in 5' hypersensitive site 3 of the human beta-globin locus control region. *EMBO Journal* **15,** 562–568. [This paper demonstrates that the position effects can be overcome by a 20 k bp DNA fragment containing the locus control region.]

Eyestone W.H. (1999). Production and breeding of transgenic cattle using *in vitro* embryo production technology. *Theriogenology* **51**, 509–517. [This paper reports a large-scale production of transgenic cattle.]

Festenstein R., Tolaini M., Corbella P., Mamalaki C., Parrington J., Fox M., Miliou A., Jones M., and Kioussis D. (1996). Locus control region function and heterochromatin-induced position effect variegation. *Science* **271**, 1123–1125. [Human CD2 locus control region (LCR) sequences are shown in this paper to be essential for establishing an open chromatin configuration.]

Forsberg E.J., Strelchenko N.S., Augenstein M.L., Betthauser J.M., Childs L.A., Eilertsen K.J., Enos J.M., Forsythe T.M., Golueke P.J., Koppang R.W., Lange G., Lesmeister T.L., Mallon K.S., Mell G.D., Misica P.M., Pace M.M., Pfister-Genskow M., Voelker G.R., Watt S.R., and Bishop M.D. (2002). Production of cloned cattle from *in vitro* systems. *Biology of Reproduction* **67**, 327–333. [The pregnancy initiation and maintenance rates of nuclear transfer embryos produced from several bovine cell types were measured to determine which cell types produced healthy calves and had growth characteristics that would allow for genetic manipulation.]

Francolini M., Lavitrano M., Lamia C.L., French D., Frati L., Cotelli F., and Spadafora C. (1993). Evidence for nuclear internalization of exogenous DNA into mammalian sperm cells. *Molecular Reproduction and Development* **34**, 133–139. [In this paper, evidence shows that the exogenous DNA is internalized into the nucleus.]

Gagne M., Pothier F., and Sirard M.A. (1991). Electroporation of bovine spermatozoa to carry foreign DNA in oocytes. *Molecular Reproduction and Development* **29,** 6–15. [In this study, electroporation was used to test the ability of spermatozoa to carry foreign DNA into the bovine oocytes.]

Gagne M., Pothier F., and Sirard M.A. (1995). Effect of microinjection time during postfertilization S-phase on bovine embryonic development. *Molecular Reproduction and Development* **41,** 184–194. [The effect of timing for DNA microinjection on DNA integration is described in this study.]

Geyer P.K. (1997). The role of insulator elements in defining domains of gene expression. *Current Opinion in Genetics and Development* **7,** 242–248. [Two models of insulator are proposed in this article: a domain boundary and a transcriptional decoy model.]

Gogolevskii P.A., Goldman I.L., Zhadanov A.B., Morzunov S.P., Klenoviyskii P.M., and Ernst L.K. (1991). Study of possibility of using restriction endonucleases to increase frequency of integration of recombinant DNA in animal genome. *Soviet Agricultural Sciences* **10,** 22–25. [This report presents the results that inclusion of restriction enzyme in DNA solution for microinjection increases the transgenic rate of rabbits.]

Gordon J.W., Scangos G.A., Plotkin D.J., Barbosa J.A., and Ruddle F.H. (1980). Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc Natl Acad Sci USA* **77,** 7380–7384. [This paper reports the first birth of transgenic mammal produced by DNA microinjection.]

Hammer R.E., Pursel V.G., Rexroad C.E., Jr., Wall R.J., Bolt D.J., Ebert K.M., Palmiter R.D., and Brinster R.L. (1985). Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* **315**, 680–683. [This paper reports the first birth of transgenic domestic animals produced by DNA microinjection.]

Haskell R.E. and Bowen R.A. (1995). Efficient production of transgenic cattle by retroviral infection of early embryos. *Molecular Reproduction and Development* **40,** 386–390. [This study explores an alternative method for producing transgenic cattle: one- to four- cell bovine embryos were exposed to a replication-defective retrovirus by microinjection of retrovirus producer cells into the perivitelline space.]

Heindorff K., Aurich O., Michaelis A., and Rieger R. (1983). Genetic toxicology of ethylenediaminetetraacetic acid (EDTA). *Mutation Research* **115,** 149–173. [This article shows the evidence that EDTA leads to morphological changes of chromatin and chromosome structure in plant and animal cells.]

Hendrickson E.A. (1997). Cell-cycle regulation of mammalian DNA double-strand-break repair. *American Journal of Human Genetics* **61**, 795–800. [This article reviews the regulation of DNA double-strand-break repair during different cell cycles.]

Higgs D.R. (1998). Do LCRs open chromatin domains? *Cell* **95,** 299–302. [This article reviews the function of LCRs in the mammalian cells.]

Horan R., Powell R., Bird J.M., Gannon F., and Houghton J.A. (1992). Effects of electropermeabilization on the association of foreign DNA with pig sperm. *Archices of Andrology* **28,** 105–114. [Using end-labeled and random primer labeled lambda HindIII DNA fragments (23–0.125 kb), it was demonstrated in this study that the DNA fragments interacted with the sperm after electroporation.]

Horvat S., Medrano J.F., Behboodi E., Anderson G.B., and Murray J.D. (1993). Sexing and detection of gene construct in microinjected bovine blastocysts using the polymerase chain reaction. *Transgenic Research* **2,** 134–140. [This paper provides a method of a PCR-based procedure for rapid bovine embryo sexing and classifying embryos for the presence of exogenous DNA.]

Houdebine L.M. (1994). Production of pharmaceutical proteins from transgenic animals. *Journal of Biotechnology* **34,** 269–287. [This article reviews animal transgenic technologies.]

Houdebine L.M. (2002). The methods to generate transgenic animals and to control transgene expression. *Journal of Biotechnology* **98,** 145–160. [This article reviews animal transgenic technologies.]

Hu W.S. and Pathak V.K. (2000). Design of retroviral vectors and helper cells for gene therapy. *Pharmacological Reviews* **52,** 493–511. [The principles and design of the modifications of retroviral vectors for gene transfer are discussed in this review.]

Humpherys D., Eggan K., Akutsu H., Friedman A., Hochedlinger K., Yanagimachi R., Lander E.S., Golub T.R., and Jaenisch R. (2002). Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. *Proceedings of the National Academy of Science USA* **16,** 16. [The results demonstrate frequent abnormal gene expression in clones, in which most expression abnormalities appear common to the NT procedure whereas others appear to reflect the particular donor nucleus.]

Hyttinen J.M., Peura T., Tolvanen M., Aalto J., Alhonen L., Sinervirta R., Halmekyto M., Myohanen S., and Janne J. (1994). Generation of transgenic dairy cattle from transgene-analyzed and sexed embryos produced *in vitro*. *Biotechnology (NY)* **12**, 606–608. [This paper reports the generation of a transgenic calf from *in vitro* produced bovine embryos which had undergone transgene analysis and sexing prior to the embryo transfer.]

Ikawa M., Kominami K., Yoshimura Y., Tanaka K., Nishimune Y., and Okabe M. (1995). A rapid and non-invasive selection of transgenic embryos before implantation using green fluorescent protein (GFP). *FEBS Letters* **375,** 125–128. [This paper reports a method of non-invasive selection of transgenic mice at the stage of preimplantation embryos.]

Jaenisch R., Jahner D., Nobis P., Simon I., Lohler J., Harbers K., and Grotkopp D. (1981). Chromosomal position and activation of retroviral genomes inserted into the germ line of mice. *Cell* **24,** 519–529. [This paper reports that the exogenous Moloney leukemia virus (M-MuLV) was inserted into the germ line of mice by exposing embryos to viruses at different stages of embryogenesis.]

Jahner D., Haase K., Mulligan R., and Jaenisch R. (1985). Insertion of the bacterial gpt gene into the germ line of mice by retroviral infection. *Proceedings of the National Academy of Science USA* **82,** 6927–6931. [This study explored the potential of retroviral vectors for transferring nonviral genes into the germ line of mice.]

Kang Y.K., Park J.S., Lee C.S., Yeom Y.I., Chung A.S., and Lee K.K. (1999). Efficient integration of short interspersed element-flanked foreign DNA via homologous recombination. *Journal of Biological Chemistry* **274**, 36585–36591. [This study investigates whether mouse short interspersed elements (SINEs) could influence the recombination frequency of foreign DNA.]

Kato M., Yamanouchi K., Ikawa M., Okabe M., Naito K., and Tojo H. (1999). Efficient selection of

transgenic mouse embryos using EGFP as a marker gene. *Molecular Reproduction and Development* **54,** 43–48. [This paper reports a reliable method that uses the EGFP (Enhanced Green Fluorescent Protein) gene as a marker for selecting transgenic embryos from preimplantation embryos.]

Kato Y., Tani T., Sotomaru Y., Kurokawa K., Kato J., Doguchi H., Yasue H., and Tsunoda Y. (1998). Eight calves cloned from somatic cells of a single adult. *Science* **282**, 2095–2098. [This paper shows that bovine cumulus and oviductal epithelial cells of the adult have the genetic content to direct the development of newborn calves.]

Katz R.A., Merkel G., Kulkosky J., Leis J., and Skalka A.M. (1990). The avian retroviral IN protein is both necessary and sufficient for integrative recombination *in vitro*. *Cell* **63**, 87–95. [This paper provides evidence that the retroviral IN protein is an integrase.]

Keefer C.L., Baldassarre H., Keyston R., Wang B., Bhatia B., Bilodeau A.S., Zhou J.F., Leduc M., Downey B.R., Lazaris A., and Karatzas C.N. (2001). Generation of dwarf goat (Capra hircus) clones following nuclear transfer with transfected and nontransfected fetal fibroblasts and *in vitro*-matured oocytes. *Biology of Reproduction* **64,** 849–856. [This paper investigates the developmental potential of caprine fetal fibroblast nuclei after *in vitro* transfection and nuclear transfer (NT) into enucleated, *in vitro*-matured oocytes.]

Keefer C.L., Keyston R., Lazaris A., Bhatia B., Begin I., Bilodeau A.S., Zhou F.J., Kafidi N., Wang B., Baldassarre H., and Karatzas C.N. (2002). Production of cloned goats after nuclear transfer using adult somatic cells. *Biology of Reproduction* **66,** 199–203. [This paper investigates the developmental potential of adult somatic nuclei after nuclear transfer (NT) into enucleated, *in vitro*-matured oocytes in a dwarf breed of goat.]

Kerr D.E., Liang F., Bondioli K.R., Zhao H., Kreibich G., Wall R.J., and Sun T.T. (1998). The bladder as a bioreactor: urothelium production and secretion of growth hormone into urine. *Nature Biotechnology* **16,** 75–79. [This paper reports the generation of transgenic mice that express human growth hormone (hGH) in their bladder epithelium, resulting in its secretion into the urine at 100–500 ng mL^{-1}.]

Krimpenfort P., Rademakers, A., Eyestone W., van der Schans A., van den Broek S., Kooiman P., Kootwijk E., Platenburg G., Pieper F., Strijker R., and De Boer H. (1991). Generation of transgenic dairy cattle using "*in vitro*" embryo production. *Biotechnology* **9,** 844. [This paper reports the first transgenic cattle produced by DNA microinjection of *in vitro* derived zygotes.]

Kubisch H.M., Larson M.A., Eichen P.A., Wilson J.M., and Roberts R.M. (1997). Adenovirus-mediated gene transfer by perivitelline microinjection of mouse, rat, and cow embryos. *Biology of Reproduction* **56,** 119–124. [This paper provides the evidence that a human adenovirus can be used to express a reporter gene transiently in nonhuman embryos.]

Kubota C., Yamakuchi H., Todoroki J., Mizoshita K., Tabara N., Barber M., and Yang X. (2000). Six cloned calves produced from adult fibroblast cells after long-term culture. *Proceedings of the National Academy of Science USA* **97,** 990–995. [This paper reports birth of six clones of an aged (17-year-old) Japanese Black Beef bull using ear skin fibroblast cells as nuclear donor cells after up to three months of *in vitro* culture (10–15 passages).]

Kumar S. and Muchmore A. (1990). Tamm-Horsfall protein–uromodulin (1950–1990). *Kidney International* **37,** 1395–1401. [This article describes the uromodulin in detail.]

Lai L., Kolber-Simonds D., Park K.W., Cheong H.T., Greenstein J.L., Im G.S., Samuel M., Bonk A., Rieke A., Day B.N., Murphy C.N., Carter D.B., Hawley R.J., and Prather R.S. (2002). Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science* **295,** 1089–1092. [This paper reports the production of four live pigs in which one allele of the alpha-1,3-galactosyltransferase locus has been knocked out.]

Lai L., Sun Q., Wu G., Murphy C.N., Kuhholzer B., Park K.W., Bonk A.J., Day B.N., and Prather R.S. (2001). Development of porcine embryos and offspring after intracytoplasmic sperm injection with liposome transfected or non-transfected sperm into *in vitro* matured oocytes. *Zygote* **9,** 339–346. [This study evaluates *in vitro* and *in vivo* development of porcine *in vitro* matured (IVM) porcine oocytes fertilized by intracytoplasmic sperm injection (ICSI) and the possibility of producing transgenic embryos and offspring with this procedure.]

Laskowski M., Jr., and Kato I. (1980). Protein inhibitors of proteinases. *Annual Review of Biochemistry*

49, 593–626. [This paper reviews the proteinase.]

Lavitrano M., Camaioni A., Fazio V.M., Dolci S., Farace M.G., and Spadafora C. (1989). Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. *Cell* **57,** 717–723. [This paper demonstrates that transgenic mice can be obtained using sperm cells as foreign DNA vectors.]

Lavitrano M., French D., Zani M., Frati L., and Spadafora C. (1992). The interaction between exogenous DNA and sperm cells. *Molecular Reproduction and Development* **31,** 161–169. [This paper provides the evidence of the presence of a specific binding protein for the DNA in sperm.]

Lavitrano M., Maione B., Forte E., Francolini M., Sperandio S., Testi R., and Spadafora C. (1997). The interaction of sperm cells with exogenous DNA: a role of CD4 and major histocompatibility complex class II molecules. *Experimental Cell Research* **233**, 56–62. [This paper reports that sperm cells from MHC class II knockout mice have a reduced ability to bind DNA compared to sperm cells from wild-type animals.]

Levings P.P. and Bungert J. (2002). The human beta-globin locus control region. *European Journal of Biochemistry* **269,** 1589–1599. [This article describes the human beta-globin locus control region in detail.]

Lin J.H., Wu X.R., Kreibich G., and Sun T.T. (1994). Precursor sequence, processing, and urothelium-specific expression of a major 15-kDa protein subunit of asymmetric unit membrane. *Journal of Biological Chemistry* **269**, 1775–1784. [This paper describes the precursor sequence, processing and *in vitro* membrane insertion properties of bovine uroplakin II (UPII), a 15-kDa major protein component of AUM.]

Magnano A.R., Giordano R., Moscufo N., Baccetti B., and Spadafora C. (1998). Sperm/DNA interaction: integration of foreign DNA sequences in the mouse sperm genome. *Journal of Reproductive Immunology* **41,** 187–196. [Sequence analysis of randomly selected clones from a library of sperm genomic DNA transformed with pSV2CAT plasmid shows that foreign sequences are integrated in a unique site of the sperm genome in this study.]

Maione B., Lavitrano M., Spadafora C., and Kiessling A.A. (1998). Sperm-mediated gene transfer in mice. *Molecular Reproduction and Development* **50,** 406–409. [This paper reports a collaborative study of sperm-mediated DNA transfer to mouse eggs in well-established laboratory conditions for *in vitro* fertilization and offspring development following embryo transfer.]

Maione B., Pittoggi C., Achene L., Lorenzini R., and Spadafora C. (1997). Activation of endogenous nucleases in mature sperm cells upon interaction with exogenous DNA. *DNA and Cell Biology* **16,** 1087–1097. [The results in this study suggest that a metabolically active process similar to apoptosis is triggered in the nuclei of mature sperm cells upon interaction with exogenous DNA.]

McCreath K.J., Howcroft J., Campbell K.H., Colman A., Schnieke A.E., and Kind A.J. (2000). Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature* **405,** 1066–1069. [This paper describes the efficient and reproducible gene targeting in fetal fibroblasts to place a therapeutic transgene at the ovine alpha1(I) procollagen (COL1A1) locus and the production of live sheep by nuclear transfer.]

McKnight R.A., Shamay A., Sankaran L., Wall R.J., and Hennighausen L. (1992). Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice. *Proceedings of the National Academy of Science USA* **89**, 6943–6947. [This study investigates whether MAR sequences from the chicken lysozyme locus, the so-called A-elements, can confer position-independent regulation to a whey acidic protein (WAP) transgene in mammary tissue of mice.]

Miller D.G., Adam M.A., and Miller A.D. (1990). Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Molecular Cell Biology* **10,** 4239–4242. [This paper provides the evidence that an unintegrated retrovirus could persist in a form that was competent to integrate after release of the block to replication.]

Milot E., Strouboulis J., Trimborn T., Wijgerde M., de Boer E., Langeveld A., Tan-Un K., Vergeer W., Yannoutsos N., Grosveld F., and Fraser P. (1996). Heterochromatin effects on the frequency and duration of LCR-mediated gene transcription. *Cell* **87**, 105–114. [This paper shows that an incomplete LCR loses this property when integrated into heterochromatic regions.]

Niemann H., Halter R., Carnwath J.W., Herrmann D., Lemme E., and Paul D. (1999). Expression of human blood clotting factor VIII in the mammary gland of transgenic sheep. *Transgenic Research* **8,** 237–247. [This paper reports the generation of transgenic sheep bearing a fusion gene construct with the human blood clotting factor VIII (hFVIII) cDNA under the transcriptional control of a 2.2 kb fragment of the mammary gland specific promoter of the ovine beta-Lactoglobulin (beta-Lac) gene.]

Niemann H. and Kues W.A. (2000). Transgenic livestock: premises and promises. *Animal Reproductive Scence* **60–61**, 277–293. [This article reviews the different aspects of transgenic study in animals.]

Otto E., Jones-Trower A., Vanin E.F., Stambaugh K., Mueller S.N., Anderson W.F., and McGarrity G.J. (1994). Characterization of a replication-competent retrovirus resulting from recombination of packaging and vector sequences. *Human Gene Therapy* **5,** 567–575. [This study shows that a replication-competent retrovirus is a novel recombinant created by exchanges between G1Na and helper sequence pPAM3.]

Paleyanda R.K., Velander W.H., Lee T.K., Scandella D.H., Gwazdauskas F.C., Knight J.W., Hoyer L.W., Drohan W.N., and Lubon H. (1997). Transgenic pigs produce functional human factor VIII in milk. *Nature Biotechnology* **15,** 971–975. [This paper reports the generation of rFVIII transgenic pigs.]

Palmiter R.D., Brinster R.L., Hammer R.E., Trumbauer M.E., Rosenfeld M.G., Birnberg N.C., and Evans R.M. (1982). Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature* **300**, 611–615. [This paper reports the generation of transgenic mice with a changed phenotype]

Patil J.G. and Khoo H.W. (1996). Nuclear internalization of foreign DNA by zebrafish spermatozoa and its enhancement by electroporation. *Journal of Experimental Zoology* **274,** 121–129. [This paper provides the direct evidence for nuclear internalization of foreign DNA by non-mammalian sperm as in mammalian sperm.]

Pemov A., Bavykin S., and Hamlin J.L. (1998). Attachment to the nuclear matrix mediates specific alterations in chromatin structure. *Proceedings of the National Academy of Science USA* **95,** 14757–14762. [This study investigates cell-cycle-dependent changes in the chromatin structure of a well characterized replication initiation zone in the amplified dihydrofolate reductase domain of the methotrexate-resistant Chinese hamster ovary cell line CHOC 400.]

Pennica D., Kohr W.J., Kuang W.J., Glaister D., Aggarwal B.B., Chen E.Y., and Goeddel D.V. (1987). Identification of human uromodulin as the Tamm-Horsfall urinary glycoprotein. *Science* **236**, 83–88. [This paper reports that uromodulin is identical to the previously characterized Tamm-Horsfall glycoprotein, the most abundant protein in normal human urine.]

Perez C.F., Botchan M.R., and Tobias C.A. (1985). DNA-mediated gene transfer efficiency is enhanced by ionizing and ultraviolet irradiation of rodent cells *in vitro*. *Radiation Research* **104**, 200–213. [This study provides the evidence that the irradiation enhances the exogenous DNA integration in cultured cells.]

Perry A.C. (2000). Hijacking oocyte DNA repair machinery in transgenesis? *Molecular Reproduction and Development* **56,** 319–324. [This article reviews the mechanism of transgene integration.]

Perry A.C., Rothman A., de las Heras J.I., Feinstein P., Mombaerts P., Cooke H.J., and Wakayama T. (2001). Efficient metaphase II transgenesis with different transgene archetypes. *Nature Biotechnology* 19, 1071–1073. [This paper demonstrates the ability of mII transgenesis to deliver large transgene efficiently.]

Perry A.C., Wakayama T., Kishikawa H., Kasai T., Okabe M., Toyoda Y., and Yanagimachi R. (1999). Mammalian transgenesis by intracytoplasmic sperm injection. *Science* **284,** 1180–1183. [This paper reports that exogenous DNA can reproducibly be delivered into an oocyte by microinjected spermatozoa.]

Phi-Van L., von Kries J.P., Ostertag W., and Stratling W.H. (1990). The chicken lysozyme 5' matrix attachment region increases transcription from a heterologous promoter in heterologous cells and dampens position effects on the expression of transfected genes. *Molecular Cell Biology* **10**, 2302–2307. [This study investigates the putative influence of the MAR located 5' of the chicken lysozyme gene on expression of heterologous genes in heterologous cell systems.]

Polejaeva I.A., Chen S.H., Vaught T.D., Page R.L., Mullins J., Ball S., Dai Y., Boone J., Walker S., Ayares D.L., Colman A., and Campbell K.H. (2000). Cloned pigs produced by nuclear transfer from adult

somatic cells. *Nature* **407,** 86–90. [This paper reports the successful production of cloned piglets from a cultured adult somatic cell population using a new nuclear transfer procedure.]

Poljak L., Seum C., Mattioni T., and Laemmli U.K. (1994). SARs stimulate but do not confer position independent gene expression. *Nucleic Acids Research* **22,** 4386–4394. [The expression of genes bounded by two SARs is consistently stimulated by about 20- to 40-fold in a pool of cell transformants, however, analysis of individual, stable cell transformants demonstrates that flanking SAR elements do not confer position-independent expression.]

Pursel V.G. and Rexroad C.E., Jr. (1993). Status of research with transgenic farm animals. *Journal of Animal Science **71** Suppl*., 10–19. [Two minimal scaffold-associated regions (SARs) from Drosophila was tested in stably transformed cells for their effects on the expression of reporter genes in this paper.]

Rosen J.M., Li S., Raught B., and Hadsell D. (1996). The mammary gland as a bioreactor: factors regulating the efficient expression of milk protein-based transgenes. *America Journal of Clinical Nutrition* **63,** 627S–632S. [This article reviews the bioreactor of transgenic animals with focus on the regulatory of gene expression in milk.]

Roth M.J., Schwartzberg P.L., and Goff S.P. (1989). Structure of the termini of DNA intermediates in the integration of retroviral DNA: dependence on IN function and terminal DNA sequence. *Cell* **58**, 47–54. [This paper reports that the proportion of the DNA with recessed ends increases during the course of infection, and the blunt ends are precursors to the recessed ends.]

Rottmann O., Antes R., Hofer P., Sommer B., Wanner G., Gorlach A., Grummt F., and Pirchner F. (1996). Liposome-mediated gene transfer via sperm cells-high transfer efficiency and persistence of transgenes by use of liposomes and sperm cells and a murine amplification element. *Journal of Animal Breeding and Genetics* **113**, 401–411. [This paper reports a method of transferring DNA into sperm cells via liposome transfection.]

Rubenstein J.L., Nicolas J.F., and Jacob F. (1986). Introduction of genes into preimplantation mouse embryos by use of a defective recombinant retrovirus. *Proceedings of the National Academy of Science USA* **83,** 366–368. [This paper reports a method of introducing transgene into embryos via a defective recombinant retrovirus.]

Ryoo Z.Y., Kim M.O., Kim K.E., Bahk Y.Y., Lee J.W., Park S.H., Kim J.H., Byun S.J., Hwang H.Y., Youn J., and Kim T.Y. (2001). Expression of recombinant human granulocyte macrophage-colony stimulating factor (hGM-CSF) in mouse urine. *Transgenic Research* **10,** 193–200. [This paper reports the first case of demonstrating biological activity of a cytokine produced in the urine of a transgenic animal.]

Schiestl R.H. and Petes T.D. (1991). Integration of DNA fragments by illegitimate recombination in Saccharomyces cerevisiae. *Proceedings of the National Academy of Science USA* **88**, 7585–7589. [The result in this paper suggests that four base pairs of homology are sufficient for some types of mitotic recombination.]

Schnieke A.E., Kind A.J., Ritchie W.A., Mycock K., Scott A.R., Ritchie M., Wilmut I., Colman A., and Campbell K.H. (1997). Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* **278,** 2130–2133. [This study shows that production of transgenic sheep by nuclear transfer requires fewer than half the animals needed for pronuclear microinjection.]

Seo B.B., Kim C.H., Yamanouchi K., Takahashi M., Sawasaki T., Tachi C., and Tojo H. (2000). Co-injection of restriction enzyme with foreign DNA into the pronucleus for elevating production efficiencies of transgenic animals. *Animal Reproductive Science* **63,** 113–122. [This paper reports whether or not co-injection of foreign DNA constructs with restriction endonuclease into the pronucleus of mouse zygotes would improve the integration frequencies of foreign DNA into the host genome.]

Shemesh M., Gurevich M., Harel-Markowitz E., Benvenisti L., Shore L.S., and Stram Y. (2000). Gene integration into bovine sperm genome and its expression in transgenic offspring. *Molecular Reproduction and Development* **56,** 306–308. [This paper demonstrates that restriction enzyme mediated insertion is an efficient method for the production of transgenic sperm and corresponding offspring by AI or embryos by IVF.]

Shin T., Kraemer D., Pryor J., Liu L., Rugila J., Howe L., Buck S., Murphy K., Lyons L., and Westhusin M. (2002). A cat cloned by nuclear transplantation. *Nature* **415**, 859. [This paper reports the first cloned cat produced by somatic cell's nuclear transfer.]

Sin F.Y., Walker S.P., Symonds J.E., Mukherjee U.K., Khoo J.G., and Sin I.L. (2000). Electroporation of salmon sperm for gene transfer: efficiency, reliability, and fate of transgene. *Molecular Reproduction and Development* **56,** 285–288. [This paper reports the investigation of uptake of exogenous DNA by electroporated salmon sperm for gene transfer.]

Soriano P., Cone R.D., Mulligan R.C., and Jaenisch R. (1986). Tissue-specific and ectopic expression of genes introduced into transgenic mice by retroviruses. *Science* **234,** 1409–1413. [This paper reports that retroviral vectors can be used for tissue-specific expression of foreign genes in transgenic mice, as well as for the identification of loci that allow developmental activation of a provirus.]

Stewart C.L., Schuetze S., Vanek M., and Wagner E.F. (1987). Expression of retroviral vectors in transgenic mice obtained by embryo infection. *EMBO Journal* **6,** 383–388. [This paper shows for the first time that the TK promoter can apparently express a gene in all tissues of adult mice and that retroviral vectors with internal promoters may provide an alternative to DNA injection for the efficient expression of genes in transgenic mice.]

Stuhlmann H., Cone R., Mulligan R.C., and Jaenisch R. (1984). Introduction of a selectable gene into different animal tissue by a retrovirus recombinant vector. *Proceedings of the National Academy of Science USA* **81,** 7151–7155. [In this study, the potential use of retrovirus vectors to transduce foreign genetic information into cells of different tissues of an animal was explored by introducing a recombinant genome carrying the Eco gpt gene into post-implantation mouse embryos.]

Swanson M.E., Martin M.J., O'Donnell J.K., Hoover K., Lago W., Huntress V., Parsons C.T., Pinkert C.A., Pilder S., and Logan J.S. (1992). Production of functional human hemoglobin in transgenic swine. *Biotechnology (N Y)* **10,** 557–559. [This paper reports that three transgenic pigs are produced using a construct containing the locus control region (LCR) from the human beta globin locus together with two copies of the human alpha 1 gene and a single copy of the human beta A gene.]

Takada T., Iida K., Awaji T., Itoh K., Takahashi R., Shibui A., Yoshida K., Sugano S., and Tsujimoto G. (1997). Selective production of transgenic mice using green fluorescent protein as a marker. *Nature Biotechnology* **15,** 458–461. [This paper reports a method to selectively produce transgenic mice using green fluorescent protein (GFP) as a marker.]

Thompson E.M., Christians E., Stinnakre M.G., and Renard J.P. (1994). Scaffold attachment regions stimulate HSP70.1 expression in mouse preimplantation embryos but not in differentiated tissues. *Molecular Cell Biology* **14,** 4694–4703. [This paper provides evidence of a limited capacity of SARs to act as insulating elements that are consistent with a proposed model of SAR-mediated chromatin opening and closing.]

Tuan D., Solomon W., Li Q., and London I.M. (1985). The "beta-like-globin" gene domain in human erythroid cells. *Proceedings of the National Academy of Science USA* **82**, 6384–6388. [This paper reports the distribution of the major and minor DNase I- hypersensitive sites in the human "beta-like-globin" gene domain.]

van Berkel P.H., Welling M.M., Geerts M., van Veen H.A., Ravensbergen B., Salaheddine M., Pauwels E.K., Pieper F., Nuijens J.H., and Nibbering P.H. (2002). Large scale production of recombinant human lactoferrin in the milk of transgenic cows. *Nature Biotechnology* **20,** 484–487. [This paper describes the production of recombinant human lactoferrin (rhLF), an iron-binding glycoprotein involved in innate host defense, at gram per liter concentrations in bovine milk.]

Van Cott K.E., Butler S.P., Russell C.G., Subramanian A., Lubon H., Gwazdauskas F.C., Knight J., Drohan W.N., and Velander W.H. (1999). Transgenic pigs as bioreactors: a comparison of gamma-carboxylation of glutamic acid in recombinant human protein C and factor IX by the mammary gland. *Genetic Analysis* **15,** 155–160. [This work provides an example of apparent differences in substrate specificity between two homologous proteins to the endogenous carboxylase of porcine mammary epithelium, which leads to varying degrees of post-translational modification.]

van der Putten H., Botteri F.M., Miller A.D., Rosenfeld M.G., Fan H., Evans R.M., and Verma I.M. (1985). Efficient insertion of genes into the mouse germ line via retroviral vectors. *Proceedings of the National Academy of Science USA* **82,** 6148–6152. [This paper presents a general strategy for the efficient insertion of recombinant retroviral vector DNA into the mouse germ line via infection of preimplantation mouse embryos.]

Velander W.H., Johnson J.L., Page R.L., Russell C.G., Subramanian A., Wilkins T.D., Gwazdauskas F.C., Pittius C., and Drohan W.N. (1992). High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. *Proceedings of the National Academy of Science USA* **89,** 12003–12007. [This paper reports the generation of transgenic pigs that produced human protein C in their milk at up to 1 g L^{-1}.]

Wakayama T., Perry A.C., Zuccotti M., Johnson K.R., and Yanagimachi R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394**, 369–374. [This paper investigates the factors governing embryonic development by introducing nuclei from somatic cells (Sertoli, neuronal and cumulus cells) taken from adult mice into enucleated mouse oocytes.]

Wall R.J. (1996). Transgenic livestock: progress and prospects for the future. *Theriogenology* **45**, 57–68. [This article reviews the transgenic technologies in animals with a focus on methodology.]

Wall R.J., Hawk H.W., and Nel N. (1992). Making transgenic livestock: genetic engineering on a large scale. *Journal of Cell Biochemistry* **49,** 113–120. [This article reviews the transgenic technologies in animal.]

Wang B., Baldassarre H., Tao T., Gauthier M., Neveu N., Zhou J.F., Leduc M., Duguay F., Bilodeau A.S., Lazaris A., Keefer C.L., and Karatzas C.N. (2002). Transgenic goat produced by DNA pronuclear microinjection of *in vitro* derived zygotes. *Molecular Reproduction and Development* **63,** 437–443. [This paper provides an efficient procedure for commercial production of transgenic goats.]

Wang B., Page R.L., and Yang, X. (1996). Improved transgenic efficiency in rabbits attributed to successful microinjection, embryo transfer and animal husbandry procedure. *Theriogenology* **45,** 342. [This paper describes an efficient procedure for making transgenic rabbits.]

Whitelaw C.B., Springbett A.J., Webster J., and Clark J. (1993). The majority of G0 transgenic mice are derived from mosaic embryos. *Transgenic Research* **2**, 29–32. [This paper provides the evidence that most G0 transgenic mice are derived from a mosaic embryo.]

Wilmut I., Schnieke A.E., McWhir J., Kind A.J., and Campbell K.H. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385,** 810–813. [This paper reports the first cloned mammal from somatic cell's nuclear transfer.]

Winegar R.A. and Preston R.J. (1988). The induction of chromosome aberrations by restriction endonucleases that produce blunt-end or cohesive-end double-strand breaks. *Mutation Research* **197,** 141–149. [This paper provides evidence that cutting frequency rather than cut end-structure is important in determining efficiency of aberration induction.]

Wright G., Carver A., Cottom D., Reeves D., Scott A., Simons P., Wilmut I., Garner I., and Colman A. (1991). High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. *Biotechnology (NY)* **9,** 830–834. [This paper describes the generation of five sheep transgenic for a fusion of the ovine beta-lactoglobulin gene promoter to the human alpha 1-antitrypsin (h alpha 1AT) genomic sequences.]

Wu X.R. and Sun T.T. (1993). Molecular cloning of a 47 kDa tissue-specific and differentiation-dependent urothelial cell surface glycoprotein. *Journal of Cell Science* **106**, 31–43. [This paper describes the biochemical differentiation of bladder epithelium.]

Xue F., Tian X.C., Du F., Kubota C., Taneja M., Dinnyes A., Dai Y., Levine H., Pereira L.V, and Yang X. (2002). Aberrant patterns of X chromosome inactivation in bovine clones. *Nature Genetics* **31**, 216–220. [This paper describes the allele-specific expression of the X-linked monoamine oxidase type A (MAOA) gene and the expression of nine additional X-linked genes in nine cloned XX calves.]

Yang X., Tian X.C., Dai Y., and Wang B. (2000). Transgenic farm animals: applications in agriculture and biomedicine. *Biotechnology Annual Review* **5,** 269–292. [This article reviews animal transgenesis with focus on biomedical application.]

Yu J., Manabe M., Wu X.R., Xu C., Surya B., and Sun T.T. (1990). Uroplakin I: a 27-kD protein associated with the asymmetric unit membrane of mammalian urothelium. *Journal of Cell Biology* **111**, 1207–1216. [This paper describes the protein composition of mammalian urothelium.]

Zani M., Lavitrano M., French D., Lulli V., Maione B., Sperandio S., and Spadafora C. (1995). The mechanism of binding of exogenous DNA to sperm cells: factors controlling the DNA uptake.

Experimental Cell Research **217,** 57–64. [The results of this study suggest that the interaction of exogenous DNA with sperm cells does not appear to be a casual event but, on the contrary, relies on a molecular mechanism based on the cooperation of specific protein factors.]

Zbikowska H.M., Soukhareva N., Behnam R., Chang R., Drews R., Lubon H., Hammond D., and Soukharev S. (2002). The use of the uromodulin promoter to target production of recombinant proteins into urine of transgenic animals. *Transgenic Research* **11**, 425–435. [This paper states that the 5.6-kb fragment of the uromodulin gene containing the 3.7-kb promoter area and, both the first exon and part of the second exon, are sufficient to provide kidney-specific expression of the lacZ gene.]

Zbikowska H.M., Soukhareva N., Behnam R., Lubon H., Hammond D., and Soukharev S. (2002). Uromodulin 0human alpha1-antitrypsin into mouse urine. *Biochemical Journal* **365**, 7–11. [This paper reports the generation of transgenic mice harbouring the regulatory sequence of the uromodulin gene to direct the expression of human alpha1-antitrypsin (alpha1AT) into urine.]

Ziomek C.A. (1998). Commercialization of proteins produced in the mammary gland. *Theriogenology* **49,** 139–144. [This article reviews the biomedical application of transgenic technology in domestic animals.]

Zoraqi G. and Spadafora C. (1997). Integration of foreign DNA sequences into mouse sperm genome. *DNA and Cell Biology* **16**, 291–300. [This study investigates the fate of the foreign DNA upon internalization into sperm nuclei.]

Biographical Sketches

Xiangzhong (Jerry) Yang was born and raised in a rural Chinese village. He received his B.Sc. degree from Beijing Agricultural University in 1982, and his M.Sc. and Ph.D. degrees at Cornell University in 1987 and 1990, all majoring in Animal Science. After a short postdoctoral training in animal embryo biotechnology, he was appointed Senior Research Associate at Cornell's Animal Science Department from 1991–1996. In June 1996, He joined the faculty of the University of Connecticut as Associate Professor of Animal Science and Head of the Biotechnology Center's Transgenic Animal Facility. In 2000, he was promoted to the rank of full professor. In 2001, Jerry was appointed as the Founding Director of the Connecticut Center for Regenerative Biology at the University of Connecticut.

In a brief career, Jerry has developed a world-renowned research program in the area of animal cloning and transgenic technology. His high-profile achievements include: (1) being the first to produce male clones from a prize Japanese breeding bull in the world in 1998. These clones were selected by the Guinness World Records as the largest clones in the world (in collaboration with his Japanese colleague, published in PNAS). (2) He was the first to produce a cloned animal from an adult farm animal (known as Amy, the calf) in the United States in June, 1999. 3) First to report that cloned animals have normal telomere lengths and have normal genetic age (published in *Nature Genetics*); 4) First to report abnormal expression of X-linked genes in cloned animals (*Nature Genetics*). Jerry has received many honors and awards including the recent Chancellor's Research Excellence Award.

Dr. Bin Wang graduated from Gansu Agricultural University in 1983, and received his B.Sc. degree in Animal Science. In 1987, he received his M.Sc. degree in Animal Science from Northwest Agricultural University. In 1994, he earned his Ph.D. degree in Biology at Nanjing University. In 1996, he completed two years of postdoctoral training at Cornell University. In 1997, he joined Nexia biotechnologies Inc. as a research associate, and was later appointed as senior scientist for transgenic animal production. Dr. Wang is an expert in transgenesis of various species of mammals, including mice, rabbits, pigs, cattle, and goats. He also has extensive experiences in animal cloning in rabbits, goats, and cattle.