Int. J. Dev. Biol. 42: 1003-1008 (1998)

The use of transgenic animals in biotechnologymouse

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ABSTRACT The development of a technique that allows for oocyte and early embryo manipulation is one of the major scientific endeavors in the field of genetic manipulation for animal disease models, basic science in gene regulation and commercial applications. Dr. Ralph Brinster is one of the most prestigious scientists in the development of this science. Through his direction and support, the undertaking of the mechanisms that are involved in the earlier stages of embryology have been productive and enlightening. This paper outlines just some of the experimental successes that evolved from Dr. Brinster's insight and mentorship of one of his pupils. The essay outlines several experimental approaches that have contributed to this field. Specifically, it addresses how the mouse oocyte and the zygote respond to messenger RNA when introduced into the cell, in comparison to comparable non-mammalian species embryos. In addition, this paper discusses some transgenic animal models, both from a basic science point of view and a commercial extension of these techniques. This extension of Dr. Brinster's pioneering work is through technology that allows for the introduction of foreign DNA that can be expressed in targeted organs, such as the mammary gland for production of pharmaceuticals for use in clinical applications.

KEY WORDS: *microinjection, embryo, transgenic animals*

Introduction

Introduction of foreign molecules into the mammalian oocyte and fertilized embryos has yielded a wealth of information as to how the female germ cell functions during development. The technology that spearheaded transgenic animal research was initially pioneered by only a few laboratories. Their approach was through direct introduction of the foreign molecules into the female germ cell of mice. Subsequent to these experiments, micromanipulation through direct injection of DNA molecules into the nucleus of the fertilized egg resulted in the production of transgenic animals. The transgenic technology was initially directed toward the laboratory mouse species to develop model systems for systemic studies, but progressed quickly to the domestic species for potential economic value. This technology, therefore, is not only important to basic science research, but also to commercial applications. One of the pioneers in this explosive field of research is Dr. Ralph Brinster, to whom this book is dedicated. This chapter outlines some of the work of just one of his pupils and is a direct result of Dr. Brinster's insight and expertise in the manipulation of the mammalian embryo.

RNA injection

Micromanipulation and microsurgical techniques allow for the alteration of the genetic makeup of mammalian embryos. Some of the earliest experiments showed that amphibian eggs could be microinjected with a number of substances without deleterious effects on development (Papaioannou and Ebert, 1986). Injected foreign mRNA was shown to be actively translated in both amphibian (Gurdon *et al*., 1971) and mouse embryos (Brinster *et al.* 1980,1981; Ebert and Brinster, 1983; Paynton *et al*., 1983). Direct injection of foreign mRNA into the mouse oocyte or fertilized egg showed significant differences between these two developmental stages primarily in the stability of the mRNA; however, the translation of the mRNA was similar (Ebert *et al.,* 1984). Experiments such as these are useful to study the fate of maternal mRNA without detectable transcriptional activity in the mature oocyte and fertilized egg. As these experiments were performed on mouse oocytes, they do not preclude the fact that there may be differences among other mammalian species yet to be explored.

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#The work outlined in the following pages is an extension of the training and support acquired while under the leadership and direction of Dr. Ralph L. Binster, a mentor and good friend for whom I owe my deepest appreciation.

0214-6282/97/$05.00
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Printed in Spain

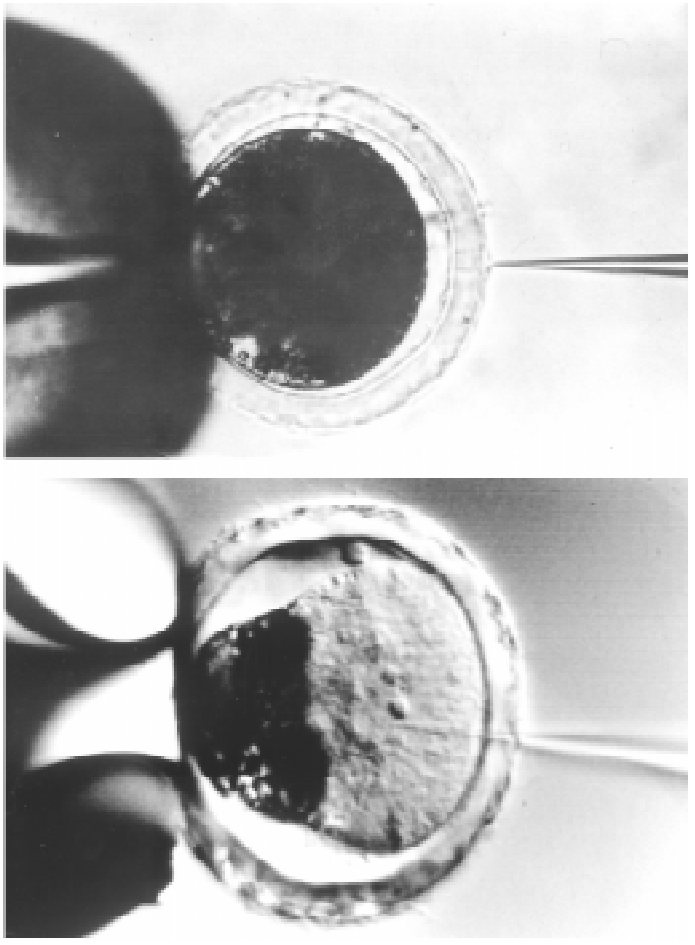

Fig. 1. Microinjection of DNA into the pronucleus of the pig zygote. (A) *A micrograph of the pig embryo which does not allow for the visualization of the pronucleus for the accurate injection of DNA into the pronucleus, which is required for the efficient integration of the foreign DNA.* **(B)** *Using the technology developed in Dr. Brinster's Laboratory, centrifugation of the zygote clearly defines the pronuclear regions and allows for the direct injection of the foreign DNA into the pronucleus without compromising the viability of the embryo.*

Injections of mitochondria

The theory that mitochondria, and thus the mitochondrial genome (mtDNA), are only maternally inherited, questions the fate of sperm mitochondria that enter the cytoplasm during fertilization (Alcivar *et al.*, 1989). The microinjection procedure was used to introduce significantly more mitochondria than would be carried into the oocyte by fertilization. The mtDNA of several mouse strains can be differentially detected by Southern blot analyses. However, microinjection of 120-150 viable mitochondria from either sperm or somatic cells failed to proliferate in the developing embryo and subsequent offspring (Ebert *et al.,* 1989). Although large numbers of mitochondria are transferred via typical cloning techniques using karyoplast fusion, to my knowledge, the maternal inheritance of mitochondria has not been disputed. Unfortunately, these experiments suggest that a mixture of mitochondrial DNA within an embryo is unlikely to be an experimental approach to genetically modify the mitochondrial genome as a transgenic targeted organelle.

DNA microinjection

In order to genetically modify an animal, direct microinjection of DNA into the pronucleus of the fertilized eggs was performed. Although the first experiments (Brinster *et al.* 1980; Gordon *et al.* 1980) showed that integration of foreign DNA was feasible, and that the integrated DNA was stable throughout development, expression of the vector was undetectable. Molecular modification of the fusion gene ultimately broke this barrier such that essentially any combination of promoter-structural gene-signal sequences can be molecularly organized to effectively result in expression of the fusion gene. The variables that are necessary for efficient integration are well documented (Brinster *et al.*, 1985). Using this technology, developed by Brinster and Gordon, many transgenic model systems have been developed (Gordon, 1983; Cuthbertson and Klintworth, 1988). Following are a few examples that my laboratory has pursued over the years. It is not meant to be exhaustive, but only emphasizes the extension of Dr. Brinster's laboratory.

My laboratory, in conjunction with Dr. Daniel Kilpatrick, has used the transgenic technology to dissect out the fundamental genetic components that are required for appropriate expression of the proenkephalin neuropeptide. Initially, transgenic mice were created to appropriately express the rat proenkephalin gene using the presumptive region that contains two distinct promoters, one of which is exclusively utilized by spermatogenic cells. Two rat *proenkephalin-chloroamphenicol acetyltransferase* fusion genes containing the two distinct promoter regions as well as 1.6 or 0.3 kb, respectively, of 5'-flanking sequence were expressed in transgenic mice.

Fusion gene transcripts in transgenic mice were found in the testes, brain and heart exclusively. Localization of the reporter gene expression in the testes was in the spermatogenic cell types which utilize the spermatogenic cell promoter (Zinn *et al.*, 1991).

TABLE 1

A SUMMARY OF 9 YEARS OF EMBRYO COLLECTIONS TO GENERATE MICROINJECTABLE GOAT EMBRYOS

Year	1989	1990	1991	1992	1993	1994	1995	1996	1997	Total
# Donors	81	76	68	104	91	115	106	50	37	728
# Ovulations	737 (9.1)	607 (8.0)	683 (10.0)	886 (8.5)	767 (8.1)	933 (8.1)	1019 (9.6)	429 (8.6)	156 (4.2)	6217 (8.5)
# Recovered (%)	485 (65.8)	349 (57.5)	379 (55.5)	567 (64.0)	527 (68.7)	623 (66.8)	678 (66.5)	248 (57.8)	121 (77.6)	3977 (64.0)
# Fertilized (%)	335 (69.1)	279 (80.0)	314 (82.8)	384 (67.7)	311 (59.0)	443 (71.1)	405 (59.7)	141 (56.9)	80 (66.1)	2692 (67.7)
1-Cell (%)	304 (90.7)	216 (77.4)	256 (81.5)	330 (85.9)	266 (85.5)	393 (88.7)	347 (85.7)	121 (85.8)	66 (82.5)	2299 (85.4)
2-Cell (%)	26 (7.8)	59 (21.1)	54 (17.2)	51 (13.3)	42 (13.5)	48 (10.8)	56 (13.8)	19 (13.5)	7 (8.8)	362 (13.4)
4-Cell (%)	5 (1.5)	4 (1.4)	4 (1.3)	3 (0.8)	3 (1.0)	2 (0.5)	2 (0.5)	1 (0.8)	7 (8.8)	31 (1.2)

Upon the development of transgenic mice that expressed the fusion gene in the appropriate regions of the brain and testes, the promoter region was altered through deletions and mutations to more accurately describe the genetic sequences and their location within the promoter region that would differentially allow for expression in only the testes.

In order to segregate the two promoters to study independent regulation and tissue specificity of the regulation of the rat *proenkephalin* gene, the upstream somatic promoter was deleted. The transgenic mice indicated that the germ-line promoter is independently functional since the reporter gene transcript was directed only to the spermatogenic cells. Analyses of the sequences within this region suggests that there are eight probable binding sites for nuclear proteins that bind specifically to the DNA in rat spermatogenic cells. This suggests that multiple *cis*-acting elements are cooperative in the regulation of the rat *proenkephalin* gene (Galcheva-Gargova *et al.,* 1993).

To find the minimal rat proenkephalin germ line promoter, a 116bp region that contained the transcriptional start site along with a proximal 51bp sequence located in the 5'-flanking region was used as a promoter for the CAT reporter gene. Analysis of the 51bp sequences corresponded to GCP1 binding element that forms cell-specific complexes with rat spermatogenic cell nuclear factors that are distinct for cyclic AMP promoter binding elements. It appears that GCP1 may contain transcriptional elements that participate during meiosis during the differentiation and development of the haploid spermatids (Liu *et al.,* 1997). These experiments outline the process of deletion and possibly a mutation that are required to effectively define the genetic elements required for normal expression of the *proenkephalin* gene.

Other experimental transgenic models have not been as rewarding. Low, Goodman and Ebert (1989) reported that the rat *somatostatin* or human *GH* fusion genes that contained the mouse metallothionein-1 (MT) promoter/enhancer sequences and the hGH 3'-flanking sequences, were selectively expressed in gonadotrophs of transgenic mice instead of somatotrophs as was predicted. In order to understand the unexpected cell-specific expression in the anterior pituitary, additional fusion genes were constructed to test separately their requirement of the MT or 3'-hGH sequences. The substitution of the 3'-hGH sequences with the simian virus 40 polyadenylation signal no longer directed strong pituitary expression in transgenic mice, but was active in the liver. Transgenic mice that contained the cytomegalovirus promoter/enhancer-*SS-hGH* fusion gene, however, showed the same high level of expression in the anterior pituitaries as the original *MT-SS-hGH* gene.

Additional laboratory animal models have also been developed. Transgenic rabbits offer an alternative to transgenic mice with the advantage of quantity of blood samples, physiological differences and size of the laboratory species.

The ability to move the transgenic technology into other mammalian species was again another accomplishment from Dr. Ralph Brinster's laboratory. The development of the first transgenic rabbits that had the hGH transgene (Hammer *et al.*, 1985a) gave us a different perspective on how gene products (GH) act within a given species. However, the generation of transgenic rabbits offered an additional laboratory model for disease studies.

My laboratory, in cooperation with Exemplar Corporation developed several lines of transgenic rabbits that expressed the human

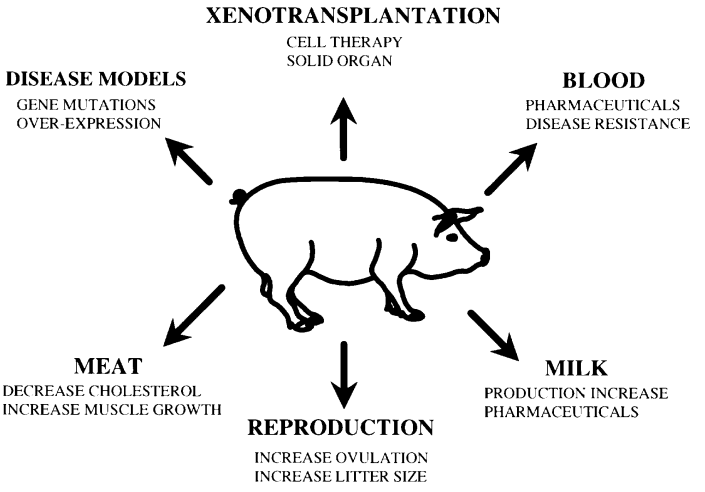

Fig. 2. Schematic diagram outlining potential areas of investigation with domestic livestock species using transgenic methodology.

CD4 protein. The studies were designed to create an AIDS model in transgenic rabbits. We set our goals to develop a model system to study HIV-1 infection and subsequent development of AIDS. The transgene contained the coding region for hCD4 with approximately 23 kb of sequence upstream of the translation start site (Gillespie et al, 1993). Microinjection of the fusion gene into rabbit zygotes resulted in 5 male and 2 female transgenic animals from 271 injected embryos. The human CD4 was expressed on the PBLs from the F1 offspring of all founder males. The preliminary studies showed that PBLs from hCD4 transgenic rabbits produced greater amounts of HIV-1 p24 antigen core protein following HIV-1 infection *in vitro* than HIV-1 p24 antigen in nontransgenic rabbits and *in vitro* cultured cells. Therefore, the transgene contains the appropriate sequences that require high-level expression in the appropriate cell type. These studies suggest that the hCD4 transgenic rabbits could have an increased susceptibility to HIV-1 infection (Snyder *et al.,* 1995).

TABLE 2

A SUMMARY OF 9 YEARS OF RECIPIENT DATA IN GOATS

Year	1989	1990	1991	1992	1993	1994	1995	1996	1997	Total
# Recipients	46	68	48	90	58	72	58	34	20	494
# Embryos Transferred	250	248	198	289	229	301	275	112	70	1972
# Pregnant	17	37	32	50	35	52	45	17	9	294
% Pregnant	37.0%	54.4%	66.7%	55.6%	60.3%	72.2%	77.6%	50.0%	45.0%	59.5%
# Live Kids	30	55	53	80	63	96	66	20	14	477
% of Embryos Transferred	12.0%	22.2%	26.8%	27.7%	27.5%	31.9%	24.0%	17.9%	20.0%	24.2%

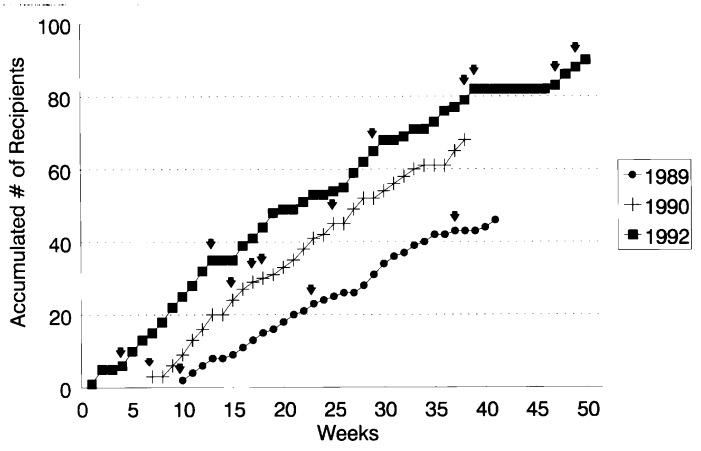

Fig. 3. A composite of three years of generating transgenic goats. *Through the use of gonadotropin treatments, we have been able to produce transgenics beyond their normal seasonal reproductive cycle.*

Microinjection of embryos from domestic species

Through the efforts of Dr. Ralph Brinster's laboratory, the technology of transferring selected genes to the genome of mice was effectively extended to other mammalian species (Hammer *et al.*, 1985b; Ebert and Schindler, 1993). The expression of these foreign genes in domestic species offered a unique opportunity to genetically modify economically important species such as pigs, sheep, and goats to produce biologically active molecules that could alter the phenotype of the transformed animals. More importantly, this phenotypic alteration is transferred from one generation to another resulting in a large population of modified animals with the same phenotype. The following are examples of how the technology of producing transgenic livestock species (goats and pigs) is being used for the production of human pharmaceuticals, and organs that may be used for xenotransplantation purposes.

The ability to effectively microinject the domestic species embryos into the pronucleus and nuclei was thwarted by the fact that the cytoplasm in pig and cattle is opaque due to lipid inclusions (Fig. 1A). However, simple centrifugation of these embryos in a microcentrifuge at 13,000g allowed for easy visualization of the nuclei (Fig. 1B). This procedure allowed the pig embryo to be targeted for genetic manipulation. The first experiments that produced transgenic pigs were performed in Dr. Ralph Brinster's laboratory using the *MT-hGH* gene construct (Hammer *et al.*, 1985b). These experiments showed that not only the fusion gene could get integrated into the genome, but that the gene could be effectively expressed in transgenic swine.

These initial experiments were the impetus for many laboratories to transgenically modify the domestic species which could be directed toward various applications in the animal industry (Fig. 2). Our laboratory developed transgenic pigs with alternative promoters driving the rat and human *GH* gene in the hope that the expression of the *GH* under physiological conditions would effectively decrease the body fat of swine, and thus generate a leaner pork product. The initial experiments were designed to use the Molony MLV rat somatotropin fusion gene. Animals that had successfully integrated the fusion gene to produce high levels of circulating rat somatotropin resulted in elevated levels of porcine insulin-like growth factor (Ebert *et al.*, 1990). Distinct from the earlier mouse model that expressed high levels of rat or human growth hormone (Palmiter *et al.*, 1982; Hammer *et al.*, 1985a), we failed to see an increase in the rate of growth of the transgenic animals. However, several phenotypic changes were evident. Fat deposition was markedly reduced as well as skeletal growth, and blood glucose levels were elevated with no signs of ketosis. The MLV promoter was shown to be effective in expression in the spleen, lung, intestine, lymph nodes, and bone marrow. These experiments showed that the MLV promoter was an effective promoter useful in high level expression of a fusion gene in swine. Although the transgenic swine had significant carcass fat reduction, unfortunately pathological studies showed several undesirable new traits associated with long bone growth and osteochondritis dissecans, a condition which is similar to acromegaly in humans. Therefore, the pathological disadvantages far outweighed the advantages of decreased carcass fat.

Additional experiments were designed to study the human cytomegalovirus (CMV) immediate early gene promoter/enhancer elements and compare these transgenic swine with the MLV promoter/enhancer elements (Ebert *et al.*, 1988,1990). These experiments were directed toward expression of the porcine growth hormone homolog in order to study the phenotypic differentiation from that of the rat or human GH transcript products. Both the MLV and CMV promoter linked to the pGH generated transgenic swine with constitutive levels of pGH throughout the life of the animal. Although some of the transgenic animals were producing only 15 times normal levels of pGH, these animals had similar adverse effects that outweighed the desired reduction in carcass fat deposition. The only difference between the two promoters was the tissue specificity of expression. The CMV promoter was expressed predominantly in the pancreas. The full potential of this technology to control body fat without altering other parameters may only be realized when it is possible to precisely regulate the expression of the microinjected genes.

The generation of transgenic dairy animals that specifically produce human proteins in the mammary tissue during lactation will generate a continually renewable source of biologically active human pharmaceuticals as an alternative to *in vitro* cell culture systems (Ebert and DiTullio, 1995). Our laboratory has specifically targeted mammary expression of human pharmaceuticals in the mammary gland of dairy goats. Goats were chosen because this dairy animal has multiple offspring, short gestation, long lactation period, and the generation of large volumes of milk.

TABLE 3

PRODUCTION OF TRANSGENIC GOATS OVER 9 YEARS

Year	1989	1990	1991	1992	1993	1994	1995	1996	1997	Total
# Recipients	46	68	48	90	58	72	58	34	20	494
# Kids Born	30	60	62	84	66	103	74	26	17	522
# Kids Transgenic	3	4	12	9	2	8	7	0	0	45
% Transgenic	10.0%	6.7%	19.3%	10.7%	3.0%	7.8%	9.5%	0.0%	0.0%	8.6%

In order to maximize the efficiency of generating transgenic goats, we implemented a hormone regime that was designed to optimize the collection and transfer of microinjectable embryos. Norgestomet ear implants were used to synchronize the estrus cycle of both the donors and recipients. Follicle stimulating hormone was the regime for superovulation of our donors, and pregnant mares serum gonadotropin was preferably used for our recipients (Selgrath *et al.*, 1990). The strategy was developed in order to synchronize the recipients with the donors without superovulation. Our data suggested that recipients that had more than 8 ovulations were less likely to maintain pregnancy than those that were within the normal ovulation parameters of between 2 and 4 ovulations (data not shown). This protocol consistently resulted in approximately 4-8 microinjectable one or two cell embryos. Thus, a typical experiment would involve 4 donors and 2 recipients that would receive 4-6 microinjected embryos. Using these parameters, our laboratory has successfully produced transgenic goats over several years. Embryo collection, recipient data, and production of transgenic animals is summarized in Tables 1, 2 and 3 respectively.

One of the most important features of our transgenic goat program was to utilize the hormonal regime beyond the normal breeding season of goats in order to continually produce transgenic animals throughout the year. Figure 3 shows that our program has produced transgenic goats from September to June and gives us an advantage over laboratories in other regions of the country that are more susceptible to warm climatic changes (i.e., Texas and Louisiana).

Using this hormonal regime, we produced the first transgenic dairy goat that expressed the human tPA (LAtPA, long acting tissue plasminogen activator) linked to the murine whey acid protein promoter (Ebert *et al.*, 1991). Enzymatically active LAtPA was produced exclusively in the mammary gland yielding a concentration of 3 μg/ml in milk with a normal lactation. Although the female founder passed the transgene to her progeny, she was later identified as a mosaic through Southern analysis of her transgenic offspring.

Although the level of expression in milk was not within the projected grams per liter that is targeted for economical levels, this transgenic animal had several lactations with no adverse effects on the mammary gland (Denman *et al.*, 1991). This female is still alive at 7 years of age.

These initial experiments, although encouraging, led to the development of additional vectors with more specific promoters for milk proteins in large domestic species such as the goat beta casein promoter (Ebert *et al.*, 1994; Ebert and DiTullio, 1995).

Xenotransplantation of organs, is presently one of the most exciting areas using transgenic domestic species (i.e., pigs). One of the most publicized aspects of the transgenic technology is in the biotechnology area.

Summary

Learning how to manipulate embryos and still maintain viability is one of the most important aspects of the biology of reproduction. However, the direct transfer of genetic material through micromanipulation has allowed investigators not only to study the physiology of embryos, but also to alter the genetic capabilities of the transformed animals. Pioneers such as Dr. Ralph Brinster have generated procedures and protocols that are now routine in many laboratories throughout the world. Using these technologies, genetically modified laboratory animals have been produced to study various aspects of animal physiology generally referred to as basic science research. The transfer of these technological breakthroughs recently has been directed towards domestic animals which may have economically important features. Understanding the interactions of gene products and regulation of the genes are paramount to the biotechnology area. Applying these technologies to agricultural species will allow for direct alterations of our domestic species to increase food production, improve feed conversion, and disease resistance. Through biotechnology, domestic animals may supply society with valuable medical compounds such as pharmaceutical drugs, antibodies, and organs for xenotransplantation to humans. Over the next few years, this technology will indeed alter agricultural science. Our thanks go to dedicated research scientists such as Dr. Ralph L. Brinster.

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