Suppression of prion protein in livestock by RNA interference

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Given the difficulty of applying gene knockout technology to species other than mice, we decided to explore the utility of RNA interference (RNAi) in silencing the expression of genes in livestock. Short hairpin RNAs (shRNAs) were designed and screened for their ability to suppress the expression of caprine and bovine prion protein (PrP). Lentiviral vectors were used to deliver a transgene expressing GFP and an shRNA targeting PrP into goat fibroblasts. These cells were then used for nuclear transplantation to produce a cloned goat fetus, which was surgically recovered at 81 days of gestation and compared with an age-matched control derived by natural mating. All tissues examined in the cloned fetus expressed GFP, and PCR analysis confirmed the presence of the transgene encoding the PrP shRNA. Most relevant, Western blot analysis performed on brain tissues comparing the transgenic fetus with control demonstrated a significant (>90%) decrease in PrP expression levels. To confirm that similar methodologies could be applied to the bovine, recombinant virus was injected into the perivitelline space of bovine ova. After *in vitro* fertilization and culture, 76% of the blastocysts exhibited GFP expression, indicative that they expressed shRNAs targeting PrP. Our results provide strong evidence that the approach described here will be useful in producing transgenic livestock conferring potential disease resistance and provide an effective strategy for suppressing gene expression in a variety of large-animal models.

nuclear transfer | short hairpin RNA | transgenic | bovine | caprine

Genetic engineering of animals and plants has played a pivotal role in research and has been directly responsible for many significant advances in agriculture and medicine. In an effort to determine the biological function of mammalian genes, embryonic stem (ES) cells can be genetically modified via homologous recombination to "knockout" single genes or specific chromosomal loci, and these modified cells can be used in the production of germ-line chimeras, which can be crossed to create null mutants (1–4). To date, the overwhelming majority of research involving the creation of genetically modified mammals has involved work with mice. Attempts to produce knockout animals in other mammalian species have been limited in large part because of an inability to derive and stably culture ES cells. Some success has been obtained by producing transgenic fetal fibroblasts via homologous recombination and then by using these cells for cloning via somatic cell nuclear transfer; however, this approach has proven to be extremely inefficient, time consuming, and costly (5–7).

RNA interference (RNAi) is a conserved biological response to double-stranded RNA, which mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids, as well as regulates the expression of protein-coding genes (8). Recent advances involving the use of RNAi-based technologies promise alternative approaches for the stable silencing of genes in a variety of different animal species including mammals. Expression constructs producing 19–29 nucleotide-inverted repeats that form short double-stranded RNA hairpins or short hairpin RNAs (shRNAs) have been demonstrated to be effective in eliciting gene silencing (9–12). This method of producing interfering RNAs enables induction of silencing by "classical" DNA expression vectors and has thus become adaptable to cell culture and the production of transgenic animals, as well as introduction into adult mammals by using established gene therapy vehicles (11). Several examples of this approach have already been reported, including recent data from our laboratory (13), demonstrating that gene constructs expressing shRNAs targeting specific genes can be stably incorporated into the genome of mouse ES cells and used to create transgenic mice exhibiting a phenotype analogous to that of the knockout animal. Importantly, this RNAi-based suppression was passed through the germ line as a dominant trait.

In more recent work, several studies involving mice have now demonstrated that lentiviral vectors can be used to deliver expression constructs encoding shRNAs into early stage embryos to produce transgenic mice in which individual genes have been targeted for silencing (14–16). Because these new methodologies neither rely on homologous recombination nor are dependent on deriving ES cells, it should be feasible to adapt this technology to the production of transgenic animals in species other than mice, including livestock.

With the recent concerns over prion-mediated diseases (transmissible spongiform encephalopathies) in livestock and their potential transmission to humans, we decided to test an RNAi-based technique for silencing the expression of the prion protein (PrP) in goats and cattle. Studies have shown that reduction of PrP expression is, in itself, sufficient to prevent infection on exposure to the pathogenic conformer of PrP. In one genetic background in mouse, loss of PrP expression gave completely normal mice with no obvious phenotype (17). In another background, PrP loss was associated with disruption of sleep cycles, but it is unclear the degree to which such a phenotype would manifest itself in other out-bred strains of mice, let alone in other species (18). Therefore, suppression of PrP by genetic engineering presents a reasonable approach for producing disease-resistant livestock and, as such, preventing transmission of prion diseases from animals to humans. In this article, we describe a strategy for using RNAi-based techniques to create a cloned transgenic goat fetus with dramatically reduced expression of PrP and present evidence that these techniques will also be adaptable to cattle. The approaches presented herein are also suitable for the creation of other types of genetically engineered animals that resist viral diseases or those that have improved agricultural traits.

Results

shRNA Design and Screening. Although the majority of recent interest in prion disease has centered on studies in cattle, the lower biosecurity requirements for studies of scrapie prompted

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Abbreviations: RNAi, RNA interference; shRNAs, short hairpin RNAs; PrP, prion protein.

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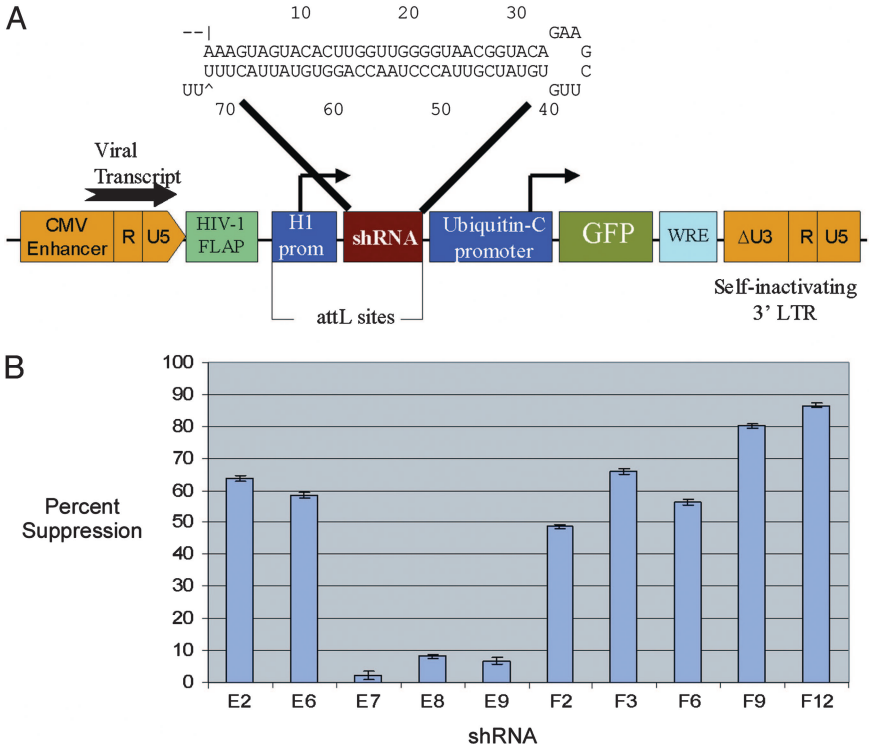

Fig. 1. Design and screening of the lentiviral shRNA expression vector. (*A*) Graphic representation of the lentiviral shRNA expression system used. This vector is a modification of the plasmid described by Lois *et al.* (23) with insertion of the mouse H1 RNase P promoter driving expression of an shRNA. The sequence shown here targets the caprine PrP mRNA (accession no. AY723292). (*B*) Percent suppression of the luciferase-PrP reporter by shRNAs targeting the PrP mRNA sequence. All data are presented as the percent reduction in luciferase activity compared with the control nonrelevant shRNA. Experiments are an average of three independent experiments, and actual percentages and standard deviations for the shRNAs are as follows: E2, 63.7 ± 0.7%; E6, 58.5 ± 0.8%; E7, 2.1 ± 1.2%; E8, 8.2 ± 0.7%; E9, 6.6 ± 1.1%; F2, 48.7 ± 0.8%; F3, 65.9 ± 0.8%; F6, 56.3 ± 0.7%; F9, 80.3 ± 0.7%; and F12, 86.5 ± 0.7%.

us to first test the possibility of using RNAi to suppress PrP expression in another commercially important livestock animal, the goat. To identify effective targeting sequences for caprine and bovine PrP, the coding sequence was processed through a computer algorithm that predicted a total of 24 shRNAs designed against the PrP mRNA (see *Materials and Methods*). Individual shRNAs were inserted into a lentiviral expression vector in which the interfering RNAs were driven by the mouse H1 RNase P promoter followed by ubiquitin C promoter-driven GFP (Fig. 1*A*). Such a strategy has been used to create transgenic mouse lines in which RNAi was used to stably suppress target gene expression (10, 13).

To facilitate screening of a relatively large number of candidate shRNAs for PrP suppression, the coding sequence for the caprine PrP was cloned downstream of the luciferase coding region in pGL3. The resulting expression vector would produce an mRNA containing the coding sequence for Firefly luciferase followed by a nontranslated sequence of the PrP mRNA. Each individual shRNA expression vector was transfected in combination with the Firefly luciferase-PrP expression plasmid and a nontargeted reporter plasmid, encoding *Renilla* luciferase, as a means of normalization. As a control, an shRNA targeting a nonrelevant sequence was transfected along with the reporter plasmids. For our initial screens, we compared the performance of these plasmids in human embryonic kidney (HEK)-293T cells using transient transfection. This screen was designed to rapidly select those shRNAs that are efficiently processed by the RNAi machinery and loaded into the RNAi-induced silencing complex (8). In accord with previous reports, such constructs allowed us to indirectly determine the relative capacity of a given shRNA to suppress PrP by monitoring luciferase activity (19). From these screens, three candidate shRNAs [F_3 with 65.9% suppression compared with the control (±0.8%), F_6 (56.3 ± 0.7%), and F_{12} (86.5 ± 0.7%)] were selected on the basis of their range of ability to suppress the luciferase reporter and used in subsequent studies (Fig. 1*B*).

Creation of Transgenic Cells for Somatic Cell Nuclear Transfer. In our initial *in vivo* trials, a characterized adult goat fibroblast cell line previously used in nuclear transfer (NT) experiments was infected with recombinant lentivirus carrying the construct encoding GFP and the PrP shRNA. Whereas a fetal fibroblast cell line may have been more optimal for cell culture, the lentiviral approach precluded any long-term culture needs. Further, the advantage of using a characterized cell line with proven ability to produce live offspring prompted us to proceed using the adult cell line. After infection, ≈30% of the cells contained the stably integrated transgene as evidenced by GFP expression (Fig. 2 *A* and *B*). These cells were selected and used for somatic cell nuclear transfer to produce cloned transgenic goat embryos, which were subsequently cultured *in vitro* to various stages of preimplantation development. In many cases, GFP expression could be visualized immediately after the nuclear transfer procedure but gradually disappeared through early embryonic divisions only to reappear at the eight-cell stage in concurrence with embryonic genome activation. Exemplary GFP-positive hatching blastocyst can be seen in Fig. 2 *C* and *D*. Because one of our goals was to determine the developmental competence of the cloned transgenic embryos, a subset was placed in culture overnight and transferred into synchronized recipient females the following day. Each doe received 12–17 one-cell stage embryos via surgical oviductal transfer. Overall, we transferred a total of 158 presumptive cloned embryos into eight recipients and obtained one pregnancy. The normal pregnancy rate for *in*

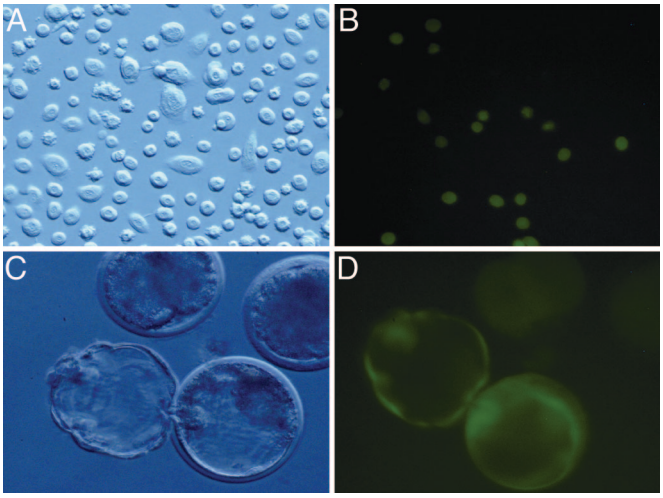

Fig. 2. GFP expression in transgenic goat fibroblasts and cloned goat embryos. (*A*) Fibroblasts are shown during preparation for somatic cell nuclear transfer. (*B*) Expression of the shRNA-GFP transgene in primary goat fibroblasts after integration of the lentiviral vector. (*C*) Bright-field image of the hatching goat blastocyst shown in *D*. (*D*) Ubiquitin C promoter-driven GFP expression in a hatching blastocyst produced via nuclear transfer by using transgenic goat fibroblasts. Goat embryos were produced by somatic cell nuclear transfer by using GFP-positive transgenic goat cells seen in *A* and *B* as nuclear donors. Note the lack of fluorescence in the nondeveloping embryos.

vivo embryo transfer is ≈60%, whereas NT rates are much lower, ≈1% survival. Thus, the pregnancy rates reported in this study are in line with studies of goat NT (20) and with the results previously recorded using this cell line (unpublished data).

Evaluation of PrP Knockdown in a Cloned Transgenic Fetus. To examine the capacity of the shRNA to silence gene expression in a disease-relevant manner *in vivo*, knockdown of the PrP had to be evaluated in the brain. Given the need to collect brain tissue and the fact that we only had one viable pregnancy, a decision was made to remove the fetus to determine whether it was transgenic and to compare PrP expression with a normal age-matched control derived by natural mating. The fetus was surgically recovered at 81 days of gestation, and tissues were harvested for analysis. A control goat fetus of similar gestational age was also harvested for comparison. All tissues derived from the cloned fetus displayed strong GFP fluorescence consistent with the presence of the transgene (Fig. 3). To confirm that the shRNA expression cassette was present in the genome, DNA was isolated from brain tissue and used as template in PCR amplifying the region between the H1 and ubiquitin promoters. As can be seen in Fig. 4*A*, the transgenic goat genome contains the shRNA expression cassette. We next assessed the ability of the shRNA to suppress PrP expression. Protein extracts were taken from transgenic and WT brain tissue and analyzed by Western blot analysis. Blots were probed by using an antibody recognizing goat PrP, which was a generous gift from Katherine O'Rourke (U.S. Department of Agricultural–Agricultural Research Service, Burns, OR) (21). As indicated in Fig. 4*B*, PrP expression was reduced by >90% in the transgenic fetus when compared with the control.

Because the lentivirus used to produce the nuclear donors is replication-deficient, it is unlikely that the transgene will be passed from the transgenic fetus to the mother; however, this possibility has not been rigorously examined. Large-animal models offer a unique opportunity in this regard because of their placental physiology. Ungulates such as the bovine and caprine concentrate their fetal placental villi together into discrete foci

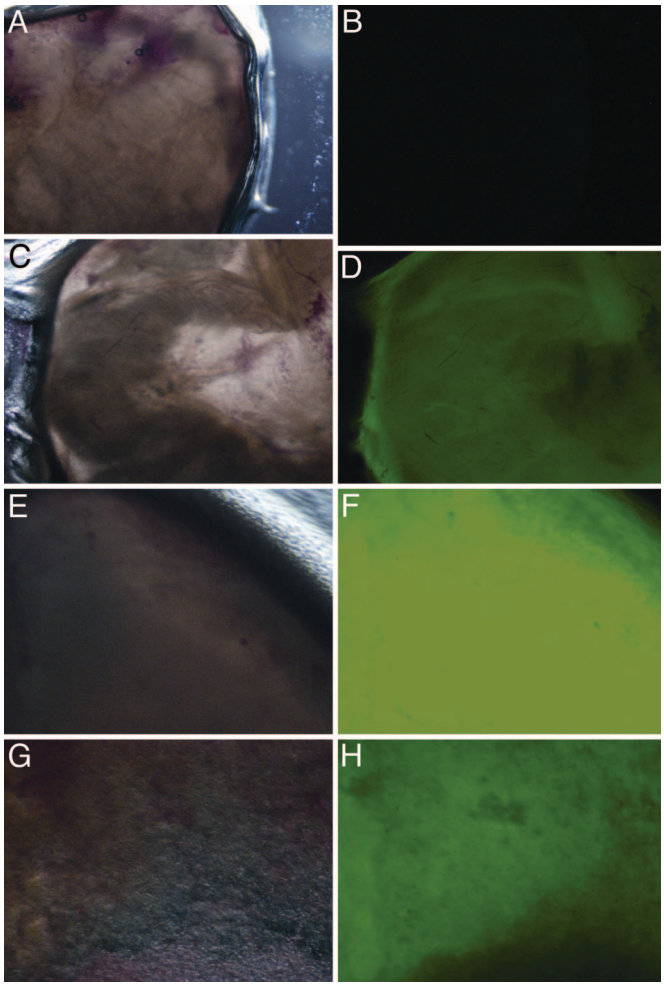

Fig. 3. Expression of green fluorescent protein in whole-mounted tissues from a cloned transgenic fetus (*C–H*). Images are transmitted (*A*, *C*, *E*, and *G*) and fluorescent (*B–D*, *F*, and *H*) light micrographs of fresh tissue samples. (*A* and *B*) Nontransgenic uterine myometrium from a recipient doe carrying a transgenic fetus. (*C* and *D*) Fetal intestinal mesentery. (*E* and *F*) Fetal intestinal lumen. (*G* and *H*) Fetal liver.

termed cotyledons. Fetal cotyledons interact with maternal regions called caruncles and together form the functional units of the placentome, where maternal-fetal nutrient exchange takes place. To determine whether the lentiviral transgene remained restricted to the transgenic fetal cells, cross-sectional tissue samples of the placentome were taken and stained for GFP expression. As can be seen in Fig. 4 *C* and *D*, GFP expression is restricted to the external fetal component, whereas the inner maternal tissue remains GFP-negative.

Creation of Transgenic Embryos by Direct Injection of Recombinant Virus Followed by *in Vitro* Fertilization and Embryo Culture. Given the effectiveness of our viral vector for delivery of transgenes into fibroblast cells growing in culture (≈30%) and because the process of animal cloning is so inefficient, we decided to test the effectiveness of our vector for delivery of the transgenes directly into early-stage bovine embryos. This strategy, based on work by Hofmann *et al.* (22) and others (23), relies on the delivery of infectious viral particles into the perivitelline space of single-cell ova and subsequent infection as the embryo develops. Due to the unusually high degree of sequence conservation between caprine and bovine PrP (96%), micromanipulation was used to inject 139 *in vitro* matured bovine ova with the same recombinant lentivirus

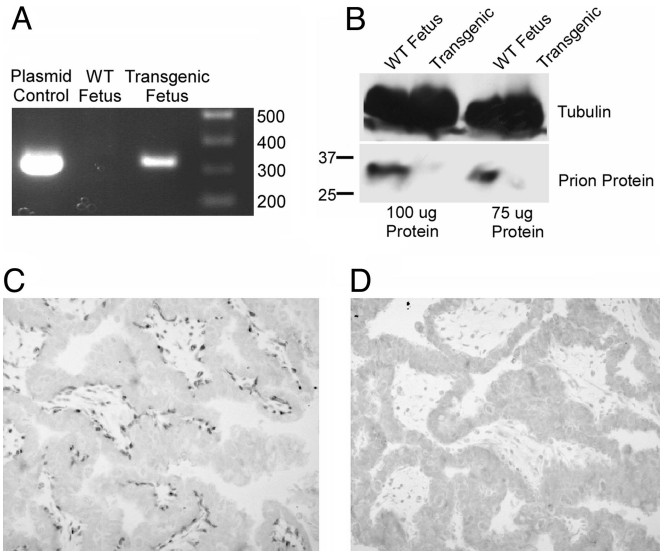

Fig. 4. Characterization of PrP suppression in the transgenic goat. (*A*) PCR amplification of the shRNA expression cassette from plasmid DNA (control), as well as genomic DNA isolated from WT and transgenic fetal tissue. (*B*) Western blot analysis of 100 and 75 μg of protein extract taken from WT and transgenic fetal brain. A residual amount of PrP can be detected in the transgenic lane; however, it is substantially reduced when compared with WT. The blot displayed was one of two independent replicates. (*C*) Immunostaining of placentome cross-sections with an anti-GFP antibody. GFP-positive transgenic cells can be seen surrounding GFP-negative maternal tissue, indicating that expression of the lentivirally delivered GFP is restricted to fetal cells. (*D*) Negative control placentome.

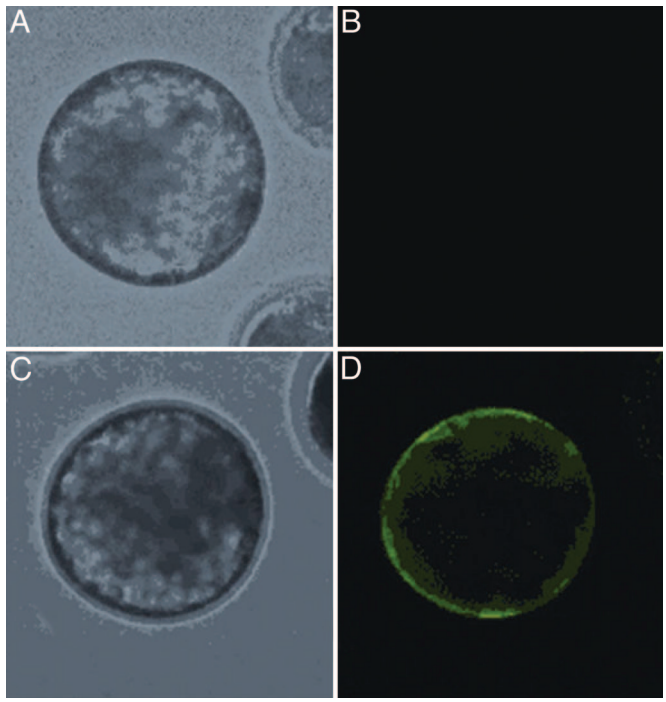

Fig. 5. Transgenic blastocysts produced by *in vitro* fertilization and embryo culture. (*A*) Bright-field image of control blastocyst (noninjected ova). (*B*) The same embryo as in *A* viewed using fluorescence microscopy. (*C*) Bright-field image of a bovine blastocyst that was produced by injection of an *in vitro* matured bovine ovum with a recombinant lentiviral vector encoding GFP and an shRNA targeting PrP, followed by *in vitro* fertilization and embryo culture. (*D*) The same embryo as in *C* viewed by using fluorescence microscopy. The expression of GFP in the embryo depicted in *D* demonstrates that this embryo has incorporated the transgene encoding GFP and a shRNA targeting PrP into its genome.

described above. After injection into the perivitelline space, the ova were fertilized and cultured *in vitro*. Of these ova, 42 (30%) developed to the blastocyst stage and 32 (76%) were GFP-positive (Fig. 5).

Expression of GFP was first observed at the morula stage of development but became much more apparent during blastocyst formation. All embryos expressing GFP exhibited uniform fluorescence throughout the inner cell mass and trophectoderm, without any obvious signs of mosaic expression. These observations indicate that the virally delivered transgene incorporated into the zygotic genome during early development and that expression initiated at the maternal zygotic transition as expected. Given the nature of our gene construct, expression of GFP is indicative that the shRNA targeting PrP was also expressed. These results strongly suggest that the methods used here are effective in the production of transgenic goats and cattle containing an expression cassette eliciting RNAi-based silencing.

Discussion

The ability to genetically engineer animals has become a standard laboratory tool for physiological, genetic, and biomedical research. However, mice represent the vast majority of transgenic animals produced to date. Additional animal models are also of critical importance for medical research because mice are not completely representative of human genetics and physiology. For example, the limited life span and small size of the mouse restricts its usefulness in studies requiring long-term evaluation of test subjects (24, 25). Thus, development of treatments and cures for human diseases are sometimes better derived from comparative studies involving animal models other than mice. In this article, we provide conclusive evidence that in a large-animal system, lentiviral delivery of shRNAs targeting specific gene(s) is indeed effective at reducing expression of the protein *in vivo*. Furthermore, these results demonstrate that lentiviral delivery of shRNA constructs has the capacity to stably knockdown genes of interest, thus providing an efficient route to functional genomic research in a large-animal model.

We acknowledge that the present study yielded only a single transgenic fetus; however, given the inefficiencies of the nuclear transfer procedure, this result is not unusual (26). Despite this inefficiency, the length of the study was significantly less compared with the time required by techniques using traditional homologous recombination. Experiments presented here as well as results by Hofmann *et al.* (22), obtained through direct injection of recombinant virus into the perivitelline space of bovine ova, clearly demonstrate the capacity to make viable transgenic bovine embryos. The use of direct viral injection to create transgenic zygotes instead of genetically modifying a cell line and producing reconstructed (cloned) embryos dramatically improves the efficiency and applicability of this technology. This technique, coupled with the ever improving shRNA expression cassettes, now enables a more functional approach to be taken when studying gene function in animal species in which stem cell technology is lacking but assisted reproductive technologies currently exist.

RNAi holds the promise of enabling production of plants and animals that are genetically altered to produce favorable characteristics. Recent years have seen a greatly increased awareness of threats posed to human health by diseases borne in livestock populations. Viral pathogens, such as the avian influenza strain H5N1, can be transmitted from domestic fowl to humans with often lethal results (27). Similarly, the detection of prion-mediated diseases in cattle have elicited the imposition of trade

restrictions and necessitated the destruction of large numbers of animals, with substantial economic impact. Although careful monitoring of animal health and appropriate safety precautions are a current approach to containing such diseases, there is theoretical potential for creating genetically engineered strains of animals with a natural resistance to numerous diseases. However, genetic methods for altering livestock have thus far been lacking.

Previous reports demonstrated that transfection of small interfering RNAs was able to reduce the level of both the endogenous and infectious PrPs in cell culture, but stable suppression of PrP in a cell line by using these small interfering RNA sequences was not demonstrated (28, 29). In this article, we report the use of shRNAs to generate both a stable cell line and a cloned transgenic goat fetus with drastically reduced expression of PrP, the causative agent of a neurodegenerative disease that might be transmitted to humans. Additionally, recent work has demonstrated RNAi to be effective in eliciting stable suppression of the foot-and-mouth disease virus as well as porcine endogenous retroviruses, both of significant concern to agriculture and studies of xenotransplantation (30–32). Whereas these methods demonstrate stable suppression in cell culture, the successful use of this technology in creating genetically modified livestock is not implicit. It is very likely that the methods described herein can be adapted to the suppression of viral sequences such as influenza, porcine endogenous retroviruses, and foot-and-mouth disease, as well as targeting genes that result in improved characteristics for the production of fiber, meat, or milk products.

Materials and Methods

Production and Screening of shRNAs. The coding sequences of the bovine and caprine mRNAs were processed through a computer algorithm that predicted a total of 24 shRNAs designed against the PrP sequence. Given the unusually high degree of sequence conservation between the bovine and caprine PrP mRNA (96%), several of the shRNAs designed perfectly matched the mRNA from both species. Individual shRNAs were ordered as antisense oligonucleotides (Sigma Genosys) and used in PCR with a sense primer homologous to the mouse H1 RNase P promoter to produce a PCR product containing the H1 promoter directly upstream of the shRNA. Reaction products were gel-purified and directionally cloned into the pENTR-D entry vector (Invitrogen) by using the recommended protocol. The shRNA sequences were verified in the Gene Technologies Laboratory at Texas A & M University. Subsequently, shRNA were inserted into a modified lentiviral expression vector (23) containing the Gateway acceptor cassette by using the Clonase reaction (Invitrogen) according to manufacturer's recommendations.

Screening of Candidate shRNAs for PrPs Suppression. Individual shRNAs were screened indirectly by monitoring the ability of each construct to silence a luciferase reporter gene containing either the bovine or caprine PrP mRNA. This system evaluates each shRNA's capacity to silence a Firefly luciferase-PrP transcript by transiently transfecting each shRNA together with the reporter gene and measuring luciferase activity by using methods described by Yu *et al.* (19). Briefly, the coding sequence for the caprine PrP mRNA (accession no. AY723292) was amplified from goat testis by using RT-PCR (Invitrogen) and cloned into the XbaI site downstream of the Firefly luciferase-coding region in pGL3 (Clontech). Individual shRNA expression vectors were transfected into National Institutes of Health human embryonic kidney 293 cells (American Type Culture Collection) by using calcium phosphate along with the Firefly-PrP expression plasmid and a nontargeted reporter plasmid, encoding *Renilla* luciferase, as a means of normalization. Luciferase activity was measured by using the Stop and Glow kit (Promega) on a luminomitor according to the standard protocol. As a control, an shRNA targeting a nonrelevant sequence was transfected along with the reporter plasmids. Human embryonic kidney 293 cells were used initially for the rapid screening because of their easy transfection and consistency in cell culture. A total of 24 shRNAs were screened in six pools containing four shRNAs each. These pools were selected for further analysis on the basis of their ability to consistently suppress the described luciferase reporter in three replicate assays. Two pools demonstrating strong suppression were expanded into their individual shRNAs and screened by using the same assay.

Production and Concentration of Viral Vectors. Lentivirus was prepared and concentrated by using methods described by Lois *et al.* (23). Briefly, viral vectors were transfected into National Institutes of Health human embryonic kidney 293 cells by using methods described above along with plasmids encoding the delta packaging signal and a vesicular stomatitis virus glycoprotein pseudotype. Medium was changed 24 h after transfection, and cells were cultured for an additional 48 h, after which medium was collected and recombinant virus was concentrated by using a standard polyethylene glycol precipitation.

Viral Infection of Fibroblasts and Nuclear Transfer. Given the previous success with producing transgenic livestock by genetically modifying fibroblasts and then using these for animal cloning, we first decided to infect goat fibroblasts by using the lentiviral shRNA construct followed by selection of transgenic cells for nuclear transfer to produce cloned embryos. Caprine fibroblasts were obtained via skin biopsy from an adult male and cultured in DMEM/F-12 with 10% FBS/0.5 mg/ml gentamycin in a humidified atmosphere of 5% CO_2/air. Cells at passage two were transferred to six-well plates (Corning). When cells achieved 50–60% confluency, fibroblast cells were infected by delivery of concentrated virus directly into the culture medium along with a 1 × polybrene solution. Cells were spun at 1,000 × *g* for 1 h and cultured in viral medium overnight. The next day, medium was changed, and cells were incubated for 3 days before assessing the expression of GFP. Once GFP expression was established (≈30% of exposed cells), cells were subpassed via standard protocol and used for nuclear transfer before passage five.

Nuclear transfer was performed as described in refs. 26 and 33. Briefly, goat oocytes were obtained from ovaries of slaughtered does and cultured *in vitro* to undergo meiotic maturation. Mature ova were enucleated, and a GFP-positive donor cell was placed in the perivitelline space. Donor cells were fused to the enucleated ova by using two dc electrical pulses (2.0 kV/cm). Recombined cells were cultured in cycloheximide for 5 h, washed, and placed in G1.3 medium (Vitrolife, Englewood, CO) in a humidified atmosphere of 5% CO_2/5% O_2/90% N_2. Cloned embryos were either transferred to the oviduct of synchronized recipient does on day 1 of culture or maintained in G1.3 for 3 days before transfer to G2.3 (Vitrolife), cultured to the blastocyst stage, and evaluated for GFP expression.

Lentiviral-Mediated Delivery of Transgenes into Bovine Zygotes. Our second approach to deliver the shRNA constructs into embryos was based on a report by Hofmann *et al.* (22). Here, injection of recombinant lentivirus into bovine ova followed by *in vitro* fertilization and embryo culture resulted in a high proportion of transgenic embryos. Bovine ova were obtained from a local abattoir and matured *in vitro*. Micromanipulation was used to inject concentrated virus into the perivitelline space of mature ova. After injection, the ova were fertilized *in vitro* by using standard procedures and then cultured *in vitro* by using G_1/G_2 embryo culture medium (20, 21) at 38.5°C in an atmosphere of

5% CO_2/5% O_2/90% N_2. After 7 days, the embryos were removed from the culture. The percentage of embryos developing to the blastocyst stage were recorded, and the embryos were visualized under a fluorescent microscope to determine whether they were transgenic as indicated by the expression of GFP.

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1. Hadjantonakis, A. K., Gertsenstein, M., Ikawa, M., Okabe, M. & Nagy, A. (1998) *Mech. Dev.* **76,** 79–90.
2. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W. & Roder, J. C. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8424–8428.
3. Pirity, M., Hadjantonakis, A. K. & Nagy, A. (1998) *Methods Cell Biol.* **57,** 279–293.
4. Zandstra, P. W. & Nagy, A. (2001) *Annu. Rev. Biomed. Eng.* **3,** 275–305.
5. Denning, C., Burl, S., Ainslie, A., Bracken, J., Dinnyes, A., Fletcher, J., King, T., Ritchie, M., Ritchie, W. A., Rollo, M., *et al.* (2001) *Nat. Biotechnol.* **19,** 559–562.
6. Kolber-Simonds, D., Lai, L., Watt, S. R., Denaro, M., Arn, S., Augenstein, M. L., Betthauser, J., Carter, D. B., Greenstein, J. L., Hao, Y., I, *et al.* (2004) *Proc. Natl. Acad. Sci. USA* **101,** 7335–7340.
7. Kuroiwa, Y., Kasinathan, P., Matsushita, H., Sathiyaselan, J., Sullivan, E. J., Kakitani, M., Tomizuka, K., Ishida, I. & Robl, J. M. (2004) *Nat. Genet* **36,** 775–780.
8. Hannon, G. J. (2002) *Nature* **418,** 244–251.
9. Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J. & Conklin, D. S. (2002) *Genes Dev.* **16,** 948–958.
10. Paddison, P. J., Caudy, A. A. & Hannon, G. J. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 1443–1448.
11. Paddison, P. J. & Hannon, G. J. (2002) *Cancer Cell* **2,** 17–23.
12. Siolas, D., Lerner, C., Burchard, J., Ge, W., Linsley, P. S., Paddison, P. J., Hannon, G. J. & Cleary, M. A. (2005) *Nat. Biotechnol.* **23,** 227–231.
13. Carmell, M. A., Zhang, L., Conklin, D. S., Hannon, G. J. & Rosenquist, T. A. (2003) *Nat. Struct. Biol.* **10,** 91–92.
14. Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L., Kopinja, J., Rooney, D. L., Ihrig, M. M., McManus, M. T., Gertler, F. B., *et al.* (2003) *Nat. Genet* **33,** 401–406.
15. Tiscornia, G., Singer, O., Ikawa, M. & Verma, I. M. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 1844–1848.
16. Tiscornia, G., Tergaonkar, V., Galimi, F. & Verma, I. M. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 7347–7351.
17. Bueler, H., Aguzzi, A., Sailer, A., Greiner, R. A., Autenried, P., Aguet, M. & Weissmann, C. (1993) *Cell* **73,** 1339–1347.
18. Sailer, A., Bueler, H., Fischer, M., Aguzzi, A. & Weissmann, C. (1994) *Cell* **77,** 967–968.
19. Yu, J. Y., Taylor, J., DeRuiter, S. L., Vojtek, A. B. & Turner, D. L. (2003) *Mol. Ther.* **7,** 228–236.
20. Shen, P. C., Lee, S. N., Wu, J. S., Huang, J. C., Chu, F. H., Chang, C. C., Kung, J. C., Lin, H. H., Chen, L. R., Shiau, J. W., *et al.* (September 12, 2005) *Anim. Reprod. Sci.*, 10.1016/j.anireprosci.2005.05.025.
21. Valdez, R. A., Rock, M. J., Anderson, A. K. & O'Rourke, K. I. (2003) *J. Vet. Diagn. Invest.* **15,** 157–162.
22. Hofmann, A., Zakhartchenko, V., Weppert, M., Sebald, H., Wenigerkind, H., Brem, G., Wolf, E. & Pfeifer, A. (2004) *Biol. Reprod.* **71,** 405–409.
23. Lois, C., Hong, E. J., Pease, S., Brown, E. J. & Baltimore, D. (2002) *Science* **295,** 868–872.
24. Almeida-Porada, G., Porada, C. & Zanjani, E. D. (2004) *Int. J. Hematol.* **79,** 1–6.
25. Porada, C. D., Park, P., Almeida-Porada, G. & Zanjani, E. D. (2004) *Fetal Diagn. Ther.* **19,** 23–30.
26. Hill, J. R., Winger, Q. A., Long, C. R., Looney, C. R., Thompson, J. A. & Westhusin, M. E. (2000) *Biol. Reprod.* **62,** 1135–1140.
27. Longini, I. M., Jr., Nizam, A., Xu, S., Ungchusak, K., Hanshaoworakul, W., Cummings, D. A. & Halloran, M. E. (2005) *Science* **309,** 1083–1087.
28. Daude, N., Marella, M. & Chabry, J. (2003) *J. Cell Sci.* **116,** 2775–2779.
29. Tilly, G., Chapuis, J., Vilette, D., Laude, H. & Vilotte, J. L. (2003) *Biochem. Biophys. Res. Commun.* **305,** 548–551.
30. de los Santos, T., Wu, Q., de Avila Botton, S. & Grubman, M. J. (2005) *Virology* **335,** 222–231.
31. Karlas, A., Kurth, R. & Denner, J. (2004) *Virology* **325,** 18–23.
32. Liu, M., Chen, W., Ni, Z., Yan, W., Fei, L., Jiao, Y., Zhang, J., Du, Q., Wei, X., Chen, J., *et al.* (2005) *Virology* **336,** 51–59.
33. Hill, J. R., Winger, Q. A., Burghardt, R. C. & Westhusin, M. E. (2001) *Anim. Reprod. Sci.* **67,** 17–26.