

Transgenesis

330 UTILIZING PRE-IMPLANTATION GENETIC DIAGNOSIS AND OPU-IVP-ET TO GENERATE MULTIPLE PROGENY OF PREDETERMINED GENOTYPE FROM CLONED TRANSGENIC HEIFERS

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Advanced reproductive technologies, comprising ovum pickup (OPU), *in vitro* embryo production (IVP), and embryo transfer (ET), were used to rapidly generate multiple F1 offspring from nine hemizygous founder-cloned transgenic (TG) heifers that highly express casein protein in their milk (Brophy B *et al.* 2003 Nat. Biotech. 21, 157–62). Moreover, embryo biopsy and genotyping were used to determine gender and presence or absence of the additional casein genes prior to ET. This minimized the number of surplus animals that would otherwise be generated from the breeding program. The aim was to generate 10 TG female (TG-F), 10 non-TG female (non-TG-F), two TG male (TG-M), and two non-TG male (non-TG-M) offspring. Some hemizygous TG bulls were required to enable the generation of homozygous transgenic animals in the next generation, while non-TG cattle would serve as controls in future studies. Transvaginal OPU was performed on non-stimulated TG heifers twice weekly for 6 weeks. Selected oocytes were matured, then fertilized *in vitro* (Day 0) with sperm from a conventional sire, and cultured to Day 6 (Thompson JG *et al.* 2000 J. Reprod. Fert. 118, 47–55). Transferable quality embryos were biopsied and cultured singularly for another 20 h, by which time genotyping results were available. On Day 7, individual embryos of the desired genotype were identified and transferred nonsurgically to synchronized recipients. Two biopsy methods were used, dependent on the stage of the embryo. For blastocysts, 10–12 trophectoderm cells were biopsied using an ultra-sharp splitting blade mounted on a micromanipulator (Herr CM *et al.* 1991 Theriogenology 35, 45–54). For morulae, the zonae pellucidae were enzymatically removed and two blastomeres per embryo aspirated with a blunt 30- μ m micropipette (Chrenek P *et al.* 2001 Theriogenology 55, 1071–81). Aliquots either from the lysed blastocyst biopsy samples or following primer extension pre-amplification of DNA (Zhang L *et al.* 1992 Proc. Natl. Acad. Sci. USA 89, 5847–51) from blastomere samples were used to determine sex and casein transgene status using multiplex PCR. Following the OPU program, the TG heifers were artificially inseminated with semen from the same sire as used for IVF; eight calved (89%). Data were compared using Fisher's exact test. The mean number of transferable Day 6 embryos produced per donor per OPU session was 1.3. Pregnancy rates following OPU-IVP-ET on Day 35 were 27/62 (44%) with a calving rate of 29%. Nine TG-F, five non-TG-F, three TG-M, and one non-TG-M were delivered vaginally at term. All calves born were of the predicted genotype. There was no significant difference in embryonic survival to term either between TG and non-TG embryos transferred, or between biopsy methods. One OPU calf died shortly after birth; the other 17 survived to weaning, as did all the AI calves. The results show that pre-implantation genetic diagnosis combined with OPU-IVP-ET can be successfully used to produce small scale-up herds from founder TG females.

331 MODIFIED SINGLE-STRANDED OLIGONUCLEOTIDE-RECOMBINASE COMPLEX MEDIATES GENE TARGETING IN MOUSE EMBRYOS

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Gene targeting is an *in situ* manipulation of an endogenous gene in a precise manner by the introduction of exogenous DNA. The process of gene targeting involves a homologous recombination reaction between the targeted genomic sequence and an exogenous targeting vector. In elucidating the function of many genes, gene targeting has become the most important method of choice. Conventional gene targeting has been achieved through the use of embryonic stem cells. However, such a procedure is often long, tedious, and expensive and has been limited in the mouse only due to a lack of usable embryonic stem cells in other species. This study was carried out to develop a much simplified procedure of gene targeting using *E. coli* recombinase recA and modified single-stranded oligonucleotides. The new procedure was attempted to modify X-linked hypoxanthine phosphoribosyltransferase (HPRT) gene. The single-stranded oligonucleotide to target exon 3 of HPRT was 74 bases in length and included three phosphorothioate linkages at each terminus (also known as S-oligo) so as to be resistant against exonucleases when introduced into zygotes. The oligonucleotide sequence was homologous to the target gene except for a single nucleotide that induces a mismatch between the introduced oligonucleotide and endogenous HPRT gene. Although the exact mechanism is yet unknown, endogenous repairing of such a mismatch would give rise to the conversion of TAT to TAG stop codon, thereby losing the function of the target gene. Prior to an introduction into zygotes, modified single-stranded oligonucleotides were preincubated with recA recombinase to enhance the homologous recombination. The recA-oligonucleotide complex was microinjected into the pronuclei of zygotes. Individual microinjected embryos that developed to the blastocyst stage were analyzed for the expected nucleotide conversion using PCR and subsequent sequencing. The conversion of TAT to TAG stop codon was confirmed in two embryos among forty tested blastocysts, so that the frequency of gene targeting was approximately 5%. The result suggests that the gene targeting was feasible by this relatively easier direct method. Subsequent transfer of gene-targeted embryos to recipients to obtain transgenic mice missing the function of HPRT gene is underway. Further technical refinement and enhancement of homologous recombination frequency will be required for the practical use of this new approach for gene targeting in mice.

332 ENHANCEMENT OF GROWTH PERFORMANCE IN DOUBLE TRANSGENIC MICE WITH GROWTH HORMONE RECEPTOR AND IGF-1 RECEPTOR GENES

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The effect of amplifying growth-related receptor signaling, through overexpression of receptors, on growth regulation in animals was examined. Transgenic mice lines were produced by DNA microinjection using the metallothionein promoter ligated to either the growth hormone receptor (GHR) or IGF-1 receptor (IGF-1R) genes (3 GHR founders and 3 IGF-1R founders). Transgenic mouse lines were estimated to contain approximately 4 to 20 copies of transgenes per cell by Southern blot analysis. Founder mice of each transgenic line transmitted transgenes into F₁ and F₂ pups with Mendelian ratio. Double transgenic (IGF-1R/GHR) mice were produced by the mating between nine pairs of IGF-1R and GHR hemizygous transgenic F₁ mice. The transmission patterns in the 78 F₂ pups produced from these matings were 20 with no transgene (25.6%), 17 with the IGF-1R gene (21.8%), 25 with the GHR gene (32.1%), and 16 with both GHR and IGF-1R genes (20.5%). The mRNA expression of transgenes using RT-PCR with the specific primers for IGF-1R and GHR genes was checked in tissues of transgenic mice. Double transgenic mice with IGF-1R and GHR genes expressed more mRNAs of transgenes than non-transgenic or single transgenic mice. Growth of double transgenic mice was fastest compared with single transgenic mice containing IGF-1R or GHR genes. And GHR transgenic mice grew faster than IGF-1R transgenic mice. When body weights of 15 transgenic mice for each transgenic line were measured at 4, 10, and 14 weeks after birth, double transgenic mice were significantly heavier compared with non-transgenic control mice at each stage (24 to 30% heavier in double transgenic mice; 15 to 20% heavier in single transgenic mice, $P < 0.05$). These results suggest that overexpression of growth-related receptor genes could promote the growth of transgenic animals with an additive effect.

333 HIGH LEVEL EXPRESSION OF BIOACTIVE RECOMBINANT HUMAN GROWTH HORMONE IN THE MILK OF A CLONED TRANSGENIC COW

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Transgenic farm animals have been proposed as an alternative to current bioreactors by expression in *E. coli* but no comparisons have been made yet for the production of the same protein by both methods. Recombinant growth hormone (hGH) was produced by fermentation in *E. coli* by a procedure routinely used for the commercial production of the hormone. Fetal fibroblasts were obtained from a Jersey 75-day fetus and transfected with separate plasmids containing constructs comprising the human growth hormone gene (650 bp cDNA) under the control of a bovine β -casein promoter and a neomycin resistance gene. Control was a nontransgenic parental cell line; all other groups were selected for 14 days with 800 μ g/mL geneticin, and three cell lines (L0–L2) were obtained and were used as donors. NT was performed as described by Salamone *et al.* 2004 Reprod. Fert. Dev. 16, 158. Two blastocysts were transferred nonsurgically per recipient cow. The blastocyst numbers per total numbers of NT by treatment were: control, 33/197; L0, 28/130; L1, 34/137; and L2, 71/470. Chi-square analysis showed no significant differences among groups. Calves born were: $n = 1, 4, 0$, and 5, from control, L0, L1, and L2 groups, respectively. The total rate of development to term of transferred doublets was 12%. After testing by PCR and Southern Blot, only one calf from line L0 showed a complete sequence for the coding region of the hGH whereas partial deletions were observed in the other animals. Artificial lactation was induced at 11 months and milking was started on Day 24 after the start of the hormonal treatment regime. Bioactivity of hGH in the milk, measured by a Nb2 cell proliferation assay, started to increase from values around 2 g/L at the onset of milking and rose steadily, reaching values of 5.0 g/L. A highly significant correlation was observed between the bioactivity and the immunoactivity determined with a specific antibody. Analysis of whey milk showed a major band corresponding to rhGH after staining with Commassie blue. This band represents about 10% of the total protein content. In comparison, in the cell extract from *E. coli*, rhGH represented less than 5%. The peptidic map of rhGH from milk was identical to that of the hormone produced by bacterial fermentation. At the present production rates, which are expected to be increased after the cow reaches a natural lactation, the annual amounts of hGH produced from this single cow would be about 4,400 g, which represents a 445% increase over our yield in conventional bacterial fermentation (500 L fermentor). Only about 15 animals would be required to cover the world needs for the treatment of children suffering from dwarfism. Here we show the production of recombinant human growth hormone in the milk of a cloned transgenic cow in levels of up to 5 g/L. The hormone has identical bioactivity to that currently produced by expression in *E. coli*. These results demonstrate that transgenic cattle can be used as an alternative for the production of this hormone.

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334 EFFECT OF CYTOPLASMIC INJECTION OF dsRNA ON TRANSIENT EXPRESSION OF THE EGFP GENE MICROINJECTED INTO RAT EMBRYOS

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RNA interference (RNAi) using double-stranded RNA (dsRNA) is a useful tool to inhibit posttranscriptional specific gene expression in mammalian cells. We investigated the effects of cytoplasmic injection of dsRNAs on transient gene expression in rat embryos when the enhanced green fluorescence protein (EGFP) gene was injected into pronuclei of zygotes. Wistar strain females were superovulated by injections of eCG and hCG and mated with males of the same strain. Pronuclear stage embryos were collected 29 h after hCG injection. The construction of the transgene, the EGFP gene controlled under the CMV-IE promoter, was dissolved at a concentration of 5 µg/mL in DNA injecting buffer (10 mM Tris-HCl, 0.1 mM EDTA), and it (3–5 pL) was microinjected into the pronuclei of embryos. After injection, embryos were cultured in KRB (Toyoda Y and Chang M C. 1974. *J. Reprod. Fertil.* 36, 9–22) at 37.0°C in 5% CO₂ and 95% humidified air until observation. In Experiment I, transient expression of the EGFP gene after microinjection into pronuclei of zygotes was examined. The EGFP expression in the embryos was observed at 6 h intervals until 48 h after the injection using fluorescence microscopy. In Experiment II, the inhibitory effect of dsRNAs targeting the EGFP mRNA was investigated. The dsRNAs for the EGFP were synthesized with T7 RNA polymerase from PCR product amplified with a pair of hybrid primers for the EGFP and T7 RNA polymerase priming site. Then the dsRNAs (1 µg/µL) solution (3–5 pL) was injected into the cytoplasm of the DNA-microinjected embryos 1 h after microinjection. As a control, the TE buffer solution (3–5 pL) was injected into the cytoplasm of the DNA-microinjected embryos. These embryos were cultured and then observed by fluorescence microscopy 48 h after DNA injection. Fluorescent intensity of the embryos was captured and analyzed with image analyzing software (Scion Image, Scion Corporation, Frederick, MD, USA), and all data were analyzed by χ^2 test. In Experiment I, the initiation of the fluorescent embryos was observed 24 h after DNA microinjection, and the proportion of fluorescent embryos in five replicates reached maximum (67%, 106/159) at 48 h after DNA injection. As shown in Table 1, there was a difference ($P < 0.05$) in the rate of fluorescent embryos between the dsRNA-injected group (18.4%, 33/179; range of 3.0–42.3%) and controls (58.8%, 77/131; range of 42.9–100%) in five replicates of Experiment II. The results of the present study suggest that injection of dsRNAs targeting the EGFP mRNA into the cytoplasm had an inhibitory effect on the transient expression of the EGFP gene microinjected into pronuclei of rat embryos. The inhibition system of the present study provides a powerful tool to study specific gene silencing, gene function, and development of early embryos.

Table 1. Effect of cytoplasmic injection of dsRNA on transient expression of the EGFP gene microinjected into rat embryos

Cytoplasmic injection	No. of ova used	% of surviving embryos	% of cleaving embryos	% of embryos with detected EGFP
Control	152	86.2	43.5	58.8
dsRNA	280	63.9*	36.4	18.4*

* Significant differences within each column ($P < 0.05$).

Ultrasonography

335 A PROCEDURE COMBINING ISTAT[®] ANALYSIS WITH OPU TO STUDY BOVINE FOLLICULAR ENVIRONMENTS

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Our previous work successfully analysed follicular fluid (45/61, 74% sampling success) from live cows using a portable clinical gas tension analyser (iSTAT Corp., Princeton, NJ). However, the oocyte recovery rate (7/24, 29%) from preovulatory follicles (POF) was disappointingly low, perhaps due to a need for the second puncture to aspirate the remaining follicular contents. This work describes a modified procedure that allowed a follicular fluid (FF) sample to be collected and stored in a gas-impermeable manner and the remaining follicular contents to be directly aspirated with or without follicular flushing from a single puncture. This modified dual port slip-luer hub, designed for use with a disposable needle ovum pick-up (OPU) system (PieMed, Netherlands), used a low negative pressure (30 mmHg), medium flow rate (25 mL/min) aspiration system with syringe flushing. FF samples for analysis were collected and stored in glass capillary tubes and the rest of the follicular contents/flushings were collected into tubes containing 10 mL collection medium and searched for oocytes. The hub's larger port, with a fitted Y connector, allows an initial flow of FF to be drawn into a capillary tube which has a polyvinylchloride (PVC) powder plugged end. When the PVC powder is permeated with FF it forms a seal effectively capping the tube. The remaining FF is diverted down the other leg of the Y connector using negative pressure. Flushing medium can be injected through the smaller port into the follicle while negative pressure is interrupted. A pilot trial examined gas tension, pH and ion concentrations from POF, dominant (D), and hormonally stimulated (S) follicles. Twenty synchronised Friesian cattle were scanned daily and follicles were tracked and mapped. The POF and D follicles were sampled on Day 20 or Day 10 respectively of the estrous cycle. The cows were then subjected to four weekly stimulations (used CIDR-B device inserted Day 2 and removed Day 5, 120 mg NIH-FSH-P1 administered twice daily on Day 5 and 6). Follicles were sampled and aspirated on Day 7. Successful FF analysis was achieved in 88% (110/125) of follicles attempted while oocyte recovery rates from