Meeting report and abstracts of the 2005 UC Davis Transgenic Animal Research Conference V

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The bi-annual UC Davis Transgenic Animal Research Conferences are held to bring together representatives from the leading laboratories doing cutting edge work on transgenic research in vertebrate animals, and those aspects of nuclear transfer-based cloning and homologous recombination that support the production of transgenic animals. This meeting fulfills the need for a small, focused meeting to bring together scientists working with transgenic non-murine animals and related technologies such as nuclear transfer-based cloning, as the problems we share cross the boundaries of the species we work with. Speakers were drawn from the leading university, government and industry laboratories in the world working on transgenic vertebrate animals, including livestock, poultry, fish and non-human primates. Approximately 100 participants attended the conference, representing 11 different countries.

Papers at this conference focused on the state-of-the-art of the science in the field of transgenic research. Presentations addressed cutting-edge methodology, such as the development of RNAi methodology to decrease the rate of non-homologous recombination (Bertolini et al.) or the use of RNAi-expressing transgenes to obtain knock-down transgenic mice (Tiscornia), both of which may lead to important breakthroughs in the production of specific types of transgenic animals across many species. Considerable progress has been made in the use of ES cells to produce transgenic chickens (van de Lavoir et al.; Han et al.) or the application of SCNT-based cloning to yield sequential rounds of gene targeting in cattle (Robl et al.). Several papers presented the current work with transgenic pigs, salmon, catfish and goats for agricultural applications, while others addressed the use of transgenic rabbits for the production of pharmaceuticals. A particular interesting talk was presented by Corrado Spadafora (Sciamanna et al.) on the role of an endogenous sperm reverse transcriptase activity in sperm-mediated gene transfer in the mouse.

The oral presentations, posters, and discussions made this an enjoyable and informative meeting, as did the bears that came to dinner on the last day. A set of speaker and poster abstracts was published in Transgenic Research 14(3): 341–360. In this issue of the journal we present the remaining abstracts.

We are looking forward to UC Davis Transgenic Animal Research Conference VI, which will again be held in the high California mountains near Lake Tahoe August 12–16, 2007. For information concerning the next conference please visit the website at http://www.biotech.ucdavis.edu/events/events.htm

Speaker Abstracts

Setting the stage: Are we there yet?

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It is now 8 years since we first met to discuss technical challenges and potential applications of transgenic animals in agriculture. Since then the advances have been staggering, yet we continue to deal with issues related to how do we change and what do we change in the genome. In this meeting both of these issues will be addressed in detail. From a technical standpoint, the goal remains the development of efficient systems for developing both random and non-random (targeted) gene modifications. While existing systems have accomplished precise and complex manipulations in the caprine, bovine and porcine genome, they are still technically challenging, and further improvements are needed. In addition, work continues on the development of alternate systems of generation transgenic animals that are simpler and less expensive, thus allowing groups with limited resources and technical expertise to explore the uses of transgenic animal technologies. Two of the methods that look particularly promising are the use of sperm-mediated transgenesis and the utilization of lentiviral vectors. While both of these technologies suffer from some drawbacks, they do offer high efficiencies and low costs as will be discussed in detail. Yet not all species of agricultural importance are made the same when it comes to generating transgenic animals. While applications in species such as fish are moving ahead with exciting and promising results, other species such as the chicken have been more difficult to manipulate, thus requiring the development of ES/EG technology. This same technology proved difficult to develop in species such as swine and cattle. Fortunately, the utilization of somatic cell nuclear transfer as a way to develop transgenic mammals of agricultural importance has obviated the need to incorporate ES or EG-based transgenic technologies in these species. Overall, however, we are approaching the time when transgenic animals of relevance in agriculture can be made economically and efficiently, and this alone will have a tremendous impact on our field as the high costs of generating these animals has precluded a high level of research in agricultural applications, and an almost absolute concentration on biomedical applications, as the latter allow for the recovery of the high research and development costs.
From a gene function perspective, however, the application of transgenic technologies in animal agricultural remain concentrated to only a few areas where we have a clear knowledge of a single or few genes affecting a trait of interest. Some of the areas that will be updated in this conference will be the modification of milk and growth properties through transgenesis. Yet, more work remains to be done to elucidate how to properly manipulate a single or a few genes and positively affect a trait of agricultural importance. An exciting example of how a single gene can have a significant impact on a complex trait is the utilization of transgenic technologies to reduce environmental impact in agriculture. This is an area of increased public concern, and application of transgenic technologies in fields that have a direct benefit to the environment and or the consumer will be critical if we are to convince the public at large of the benefits these technologies have for dealing with issues of societal importance. As we get close to the 10 year anniversary of the first Granlibakken conference, we can predict a merging of technical advances, and an increase in our knowledge of how genes affect traits of interests, so that transgenic animals with obvious societal benefits through reduced environmental impact, increased disease resistant, adaptability to unique environmental condition, or increased nutritional value can be generated.

Are we there yet? Not quite. But we are much closer than we were eight years ago.

A central role of sperm endogenous reverse transcriptase activity in sperm-mediated gene transfer

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Sperm-mediated gene transfer (SMGT) procedures are based on the well-established ability of spermatozoa to spontaneously bind exogenous DNA molecules and deliver them to embryos at fertilization. However, contradictory results on the final fate of the foreign sequences, published by numerous laboratories in a variety of animal species, leave the unanswered question of whether exogenous sequences are integrated in host genomes or persist as extrachromosomal structures. We have been studying the underlying molecular mechanisms of SMGT for many years. A turning point in our studies has come from the discovery, in mouse spermatozoa, that an endogenous reverse transcriptase (RT) activity can reverse-transcribe sperm-bound exogenous RNA molecules into cDNA copies. To test the functional role of that activity, we have carried out in vitro fertilization (IVF) assays using spermatozoa that had been preincubated with RNA from a beta-galactosidase (beta-gal) gene-containing construct: we have found that beta-gal RNA was taken up by sperm cells, reverse-transcribed, transmitted to embryos and propagated in a mosaic pattern to founder animals and further to F1 progeny. Expression of the beta-gal protein was also detected in tissues of both F0 and F1 animals. Thus, the sperm endogenous RT can generate biologically active reverse-transcribed cDNA copies of foreign RNA. In further support of this conclusion, we have designed a set of experiments in which spermatozoa were incubated with a plasmid harboring a green fluorescent protein (EGFP) retrotransposition cassette prior to IVF. We have found that reverse-transcribed EGFP DNA sequences are generated and transmitted to embryos in IVF assays. After implantation into foster mothers, these embryos developed into viable mice that expressed EGFP.

These results indicate that an efficient machinery is present in murine spermatozoa, which can reverse-transcribe exogenous RNA molecules, and can also sequentially transcribe and reverse-transcribe foreign DNA so as to generate transcriptionally competent ‘retro-genes’. These retro-genes are not integrated in the host genomes, can be transmitted to offspring in a non-Mendelian way and are expressed as newly acquired genetic traits in the tissues of adult animals. An endogenous RT activity is also present in swine and human spermatozoa, suggesting that it is a general phenomenon not restricted to the murine system. In conclusion, these results suggest that SMGT is a retrotransposon/retroviral machinery-dependent phenomenon.

Efficient transgene delivery using lentiviral vectors

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Transgenic livestock are an exciting adjunct to classical genetic approaches to understand and exploit biological variation. The technology overcomes the limitations of classical animal breeding regimes, where importation of genes by crossbreeding is limited to those traits already present within a given species. In addition it offers the potential to develop novel biotechnological applications, including models of human disease and drug development. New methods including the use of viral vectors to deliver transgenes have been developed. These vectors offer an advantage over more standard methodology in that they enable spectacularly efficient generation efficiencies. Nevertheless they are limited in specific aspects. The pros and cons of lentiviral transgenesis will be presented with an update of progress with this exciting transgene delivery system.

The Enviropig™: phytase nutrition, physiology and tissue composition

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The Enviropig™ is a genetically enhanced pig that secretes phytase in its saliva. This characteristic enables digestion of phytate phosphorus in the diet, which eliminates the need for
dietary supplementation with phytase and substantially decreases the concentration of phosphorus in the manure. The expressed phytase, which was purified and characterized from three different lines of the Enviropig™ breed, exhibited catalytic properties identical to that of the original *E. coli* enzyme despite being glycosylated at several sites. The fifth generation of the Cassie line of pigs produced salivary phytase at a level similar to that of the original founder pig. The growth and reproductive characteristics of the pigs are similar to that of the comparator Yorkshire pigs. Data from a growth trial and tissue analysis of the Cassie line provided comparative data for boars and gilts from both the Enviropig™ breed and the comparator Yorkshire breed, including hematology and clinical biochemistry at the weaning, growing and finishing phases of growth, as well as finishing phase organ weights, commercial cuts, physical characteristics and chemical composition of the major tissues. Immunohistochemical analyses for the phytase enzyme in tissue sections and enzyme assays from four different lines of the new breed have shown that the bulk of the phytase is produced in the parotid, submaxillary and sublingual glands with localized expression of phytase in pharyngeal tissues. However, neither enzyme protein nor enzyme activity was detected in major tissues, for example, muscle, liver, kidney or fat. Data collected on the Enviropig™ breed have either been not significantly different (*P* > 0.05) or, where different, were usually within the normal ranges reported for the Yorkshire breed. Genomic and proteomic analyses are in progress.

**Endogenous production of omega-3 fatty acids in milk**

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Omega-3 (n-3) polyunsaturated fatty acids (PUFA) are essential components of the diet, and are well documented for their beneficial effects on cardiovascular well-being. Since vertebrates lack the omega-3 fatty acid desaturase responsible for synthesizing n-3 PUFA, and because n-3 and n-6 PUFA are not interconvertible in mammals, n-3 PUFA can only be obtained through dietary sources. As a result of increased consumption of vegetable oils rich in n-6 fatty acids, and the reduced consumption of fish and other foods rich in n-3 fatty acids, typical Western diets contain n-6:n-3 PUFA ratios that are greater than 10. Health studies suggest that a dietary n-6:n-3 PUFA ratio closer to 5 would be more favorable for cardiovascular well-being. Increasing the n-3 PUFA of beef and dairy products would provide a compelling example of how biotechnology could be employed to produce functional foods for the enhancement of human health.

*Caeorhabditis elegans* synthesizes a wide range of PUFAs, and possesses the only known examples of delta-12 and omega-3 fatty acid desaturase enzymes in the animal kingdom. The cDNA coding sequences of the *C. elegans* delta-12 and omega-3 fatty acid desaturases were each placed under the control of cellular phospholipid revealed significant decreases in MUFA and arachidonic acid (AA, 20:4n6), and increases in linoleic acid (LA, 18:2n6), alpha-linolenic acid (ALA,18:3n3) and eicosapentaenoic acid (EPA, 20:5n3). The fatty acid composition of triacylglycerols derived from transduced cells was similarly, but less dramatically, affected (Morimoto et al., *J Dairy Sci* 88(3): 1142–1146, 2005) These results demonstrate the functionality of *C. elegans* fatty acid desaturase enzymes in mammalian cells.

Transgenic mice expressing the *C. elegans* omega-3 desaturase under the control of a lactation-induced mammary gland promoter were produced by pronuclear microinjection. Mid-lactation milk was collected and the PUFA composition of milk triacylglycerides and phospholipids were analyzed. A significant decrease in n-6 PUFA (LA, AA), concomitant increase in n-3 PUFA (ALA, EPA), and an overall decrease in the n-6:n-3 PUFA ratio was observed in the phospholipid fraction of milk produced by transgenic omega-3 desaturase mice (Kao et al., *XIII Int. Plant & Animal Genome Meeting*; http://www.intl-pag.org/pag/13/abstract/PAG13.P763.html). The brain fatty acid composition of pups weaned from transgenic omega-3 fatty acid desaturase dams revealed significantly elevated levels of EPA and docosahexaenoic acid (DHA, 22:6n3) fatty acids relative to pups weaned from nontransgenic control dams fed the same diet.

**Properties of milk from human lysozyme transgenic goats**

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Human milk is a prime source of factors other than antibodies that help to fight disease and infection in young children. The antimicrobial compounds lysozyme and lactoferrin are two of the primary components involved. While present in human milk throughout lactation, lysozyme and lactoferrin are present at appreciable levels in the milk of dairy animals only at times of infection. Our work is directed at altering the properties of milk by adding genes to benefit the lactating animal itself as well as the resulting animal food products. Genetically engineering ruminant milk to produce significant amounts of these compounds throughout lactation has the potential to offer benefits on several levels, including those to the producer and consumer of milk as well as to animal health and well-being. We have generated transgenic dairy goats that express human lysozyme in their milk at 68% of the level normally found in human milk. The transgene is stably inherited, and standard milk production parameters fall within the range of our non-transgenic control animals. Preliminary *in vitro* data suggest that the inhibitory effects on bacterial growth realized with the human lysozyme – containing milk from our transgenic animals exceed the effects observed by spiking control goats’ milk with commercially available, active human lysozyme. Further preliminary data show that the consumption of pasteurized milk from our human
sequential gene targeting system used in this study alleviates the need for germline transmission to produce complex genetic modifications and should be broadly applicable for gene functional analysis and for biomedical and agricultural applications.

Production of transgenic birds through primordial germ cells

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Avian embryonic stem cells derived from Stage X chicken embryos can be maintained in culture indefinitely. The cells can be transfected with small and large constructs and remain pluripotent as evident by extensive contribution to all somatic tissues after injection into recipient stage X embryos. After evaluating > 60,000 offspring, however, no germine transmis-sion of the injected ES cells has been obtained. We therefore, searched for other cell types that would be able to colonize the germine after long-term culture.

Avian primordial germ cells (PGCs) migrate through the vasculature from the germinal crescent to the genital ridges. During this migration PGCs can be collected from the blood and when injected into the vasculature of a recipient embryo they contribute to the germine. Normally, primordial germ cells when kept in culture, change to an embryonic stem cell phenotype, called an embryonic germ (EG) cell. Primordial germ cells were collected from the blood of stage 14–16 embryos and seeded into 96 or 48 wells containing a feeder layer. The PGCs were grown in conditioned KO-DMEM supplemented with growth factors. Surprisingly, PGCs remained visible after extended culture and could be expanded as PGCs.

A total of 12 PGC cell lines have been derived and three cell lines were injected into the vasculature of Stage 13–16 (H&H) recipient embryos. All of these lines colonized the germine as evident by germine transmis-sion. All 18 roosters tested transmitted through the germine and 12 of 18 birds transmitted through the germine at ≥10%.

Embryonic germ cell lines have been derived from the cultured PGCs. These cells look and behave quite similarly to embryonic stem cells; when injected into a recipient embryo the EG cells contributed to the somatic tissues but not the germine.

Chicken pluripotent cells and transgenesis

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Research on pluripotent cells derived from embryos and fetus has a great impact on developing innovative technologies in various fields of life science including agriculture, medicine, pharmaceutics and biotechnology. Stem cells in various species have been established since the first isolation of mouse stem cells.
succeeded, and great anticipation on their contribution to developing novel medicine and biotechnology is now made. Pluripotency is the most important and critical characteristic of stemness, and numerous parameters are used to confirm the pluripotency. However, full-term development of stem cells transplanted into embryos, which is the ultimate measure of pluripotency, has been reported primarily in the mouse. We have continuously made efforts to establish stem cells in the avian species and finally made good progress to establish embryonic germ (EG) cells derived from primordial germ cells (PGCs) in the embryonic gonads. The established EG cells were pluripotent, and germline transmission was confirmed after transfer of EG cells into recipient embryos. Recent progress of research on EG cells established that new markers for chicken EG cells was established, which enabled quick detection of PGC or EG cells. The established PGCs and EG cells are now being employed for a stem cell-mediated germline transmission system for mass production of transgenic animals and discovery of an alternative source of stem cells in aves.

The development of transgenic chickens to produce human pharmaceuticals

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The limited production capacity available to the biopharmaceutical industry has led many companies to explore alternative manufacturing platforms including transgenics. GeneWorks has developed a gene delivery system that consistently produces transgenic chickens without the significant silencing of the transgene that is often seen in other systems. Our system uses a viral vector based on the human immunodeficiency virus, type 1 (HIV-1) to introduce the gene of interest into the stage X embryo of unincubated fertile chicken eggs. The viral DNA will integrate into the genome of each cell differently, or not at all, creating a chimeric hatching typically called “mosaic”. At maturity, semen samples from the mosaic roosters are collected and analyzed for the vector DNA by PCR. The roosters with vector-positive semen are then bred to normal hens, and the blood from day-old chicks is analyzed by PCR to identify the hemizygous transgenic founder (G0) offspring. Approximately 50% of the semen-positive mosaic roosters will produce transgenic hatchings with a frequency of 0.2% to 4.0%. In our initial pilot project, one rooster produced two different genetic lineages, and one of these lines has been carried through four generations (G4). In these birds, the gene encoding eGFP is driven by the phosphoglycerol kinase promoter, and the protein has been shown to be expressed ubiquitously and throughout development. However, this is a nonsecreted protein so the protein levels in the egg cannot be measured. Subsequently, seven mosaic roosters have produced 34 G0 offspring for three separate human genes including two pharmaceutical products. These genes are also driven by ubiquitous promoters, and the expression levels for these proteins in the blood are as high as 1 mg/ml. If this level of protein is maintained in the egg, transgenic chickens coupled with GeneWorks’ established expansion and production infrastructure are a viable alternative for manufacturing biopharmaceuticals.

Improving the efficiency of homologous recombination

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The ability to manipulate the genome of mammalian cells by gene targeting is a powerful tool in transgenesis, gene therapy, and functional genomics. The low efficiency of gene targeting in comparison with non-homologous recombination events (random integration) limits its practical applicability. In eukaryotes, two major DNA recombination pathways have been defined, the non-homologous end joining (NHEJ) and the homologous recombination (HR) pathways. In general, exogenous DNA integration in vertebrate cells occurs at a higher frequency via random or illegitimate events (NHEJ) than by HR. One possible way to reverse this process is to manipulate the cellular recombination machinery to improve gene targeting efficiency. To increase our understanding of the contribution of the NHEJ pathway to recombination and DNA integration processes, RNAi was used to down-regulate transiently key proteins involved in the NHEJ machinery in human HCT116 cells. Transfection of gene-specific siRNA to NHEJ proteins resulted in down-regulation of the targeted protein to approximately 20% of control levels 48 h after transfection, with recovery to pre-treatment levels by 96 h. The biological effects of the NHEJ pathway down-regulation on cell cycle and survival, DNA integration, and frequency of HR were characterized by (a) radiosensitivity, (b) random and (c) homologous DNA integration efficiency 48 h after RNAi treatments. The RNAi-induced down-regulation of key components of the NHEJ pathway impaired the ability of cells to repair DNA damage. There was a significant shift in the proportion of cells accumulating at the G2 checkpoint and greater sensitivity to radiation in RNAi-treated cells. A significant decrease in random DNA integration was detected in treated cells compared with controls.

Transgenic knockdown mice using siRNA mediated viral transgenesis

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Lentiviral vectors have emerged as powerful tools for both basic and applied research. They combine high carrying capacity, robust production and the ability to transduce a wide range of cell types, including non-dividing cells. We have designed lentiviral vectors capable of expressing shRNAs from both constitutive and regulatable polIII promoters, and used them both in vitro or in vivo to specifically down-regulate target genes. Lentiviral vectors can transduce ES cells and early embryos, and due to the fact that the vector integrates into the host genome, lentiviral transgenesis results in stable, long-term expression of the expression cassette. We are using lentiviral
Production of two vaccinating recombinant rotavirus proteins in the milk of transgenic rabbits

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Rotavirus are the main cause of infantile viral gastroenteritis worldwide leading to approximately 500,000 deaths each year, mostly in the developing world. For unknown reasons, live attenuated viruses used in classical vaccine strategies were shown to be responsible for intussusceptions (a bowel obstruction). New strategies allowing production of safe recombinant non-replicating rotavirus candidate vaccine are thus clearly needed. In this study we utilized transgenic rabbit milk as a source of rotavirus antigens. Individual transgenic rabbit lines were able to produce several hundreds of micrograms per ml of secreted recombinant VP2 and VP6 proteins in their milk. Viral proteins expressed in our model were immunogenic and were shown to induce variable reduction in viral antigen shedding after challenge with virulent rotavirus in the adult mouse model according to the route of administration. The protocols used were (i) systemic injection of whole milk (ii) gavage with whole milk (iii) rectal inoculation of partially purified VP2 and VP6. Anti-viral protein antibodies (IgG and IgA) were obtained in all cases and in 100% of the animals. The protection rate of the mice immunized following the three protocols and infected by the virus was 22, 28 and 98%, respectively. To our knowledge, this is the first report of transgenic mammal bioreactors allowing the rapid co-production of two recombinant viral proteins in milk to be used as a vaccine. Several kilograms per year of the viral proteins implying a few hundreds rabbits could be sufficient to vaccinate a significant proportion of the people at risk.

Expression of a humanized antibody repertoire in transgenic rabbits

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Polyclonal antibodies from human and animal plasma are efficacious and safe therapeutics for the treatment of many disease indications. To eliminate the dominant side effect of currently available polyclonal products from animals, namely serum sickness caused by the immune response of the patient against animal immunoglobulins, we have generated genetically engineered rabbits producing humanized immunoglobulins.

All animals produce antigen-specific antibodies by a two-phase process. First, a primary antibody repertoire is created by recombining V, D, and J immunoglobulin gene segments. In mice and humans this step results in considerable diversity as 100s of VDJ genes are randomly recombined and genes are imprecisely joined together. However, in most other vertebrates, including rabbits and chickens, this first step of VDJ recombination does not lead to significant diversity, because only a limited number of genes are employed. In addition, immunoglobulin gene rearrangement stops early in live virus immunization. To enhance diversity of the primary repertoire, rabbits and chickens use a second step to modify antigen-binding regions through templated (gene conversion) and non-templated (hypermutation) mutational processes. Gene conversion creates broad diversity by modifying all three antigen-binding sites of the VDJ region.

For the expression of a diversified human antibody repertoire in rabbits we have developed a novel genetic engineering approach based on humanized rabbit immunoglobulin loci. First, large parts of rabbit immunoglobulin loci were isolated from BAC libraries and sequenced. These sequences then served as a template to introduce human immunoglobulin gene segments by homologous recombination in E. coli. Only gene segments encoding variable and constant regions of human antibodies were introduced, while all control regions (i.e. promoters, enhancers, recombination signals, etc.) were left unchanged. The humanized immunoglobulin loci were introduced into pronuclei of fertilized oocytes for production of transgenic rabbits. To ensure the preferential production of human immunoglobulin over endogenous gene products, animals were crossed with natural strains of rabbits that have specific mutations in their immunoglobulin genes, and therefore, impaired expression of endogenous loci.

Characterization of transgenic rabbits demonstrated production of a diversified human antibody repertoire. Transgenic immunoglobulin loci were diversified by gene conversion in rabbits but not in mice. These results demonstrate that humanized rabbit immunoglobulin loci allow efficient expression of a human antibody repertoire in a gene-converting animal.

Health and survival of cloned calves

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Cloning technology offers new possibilities for agriculture, therapeutic and regenerative medicine, transgenic animals, and the study of fundamental developmental processes. Application of this technology is limited at present by inefficiencies of the nuclear-transfer process, high rates of abnormal pregnancies and fetal loss, and concerns about the health and longevity of resulting animals. Reports of 369 term cloned calves published in peer-reviewed journals by nearly 20 different laboratories
over a 5-year-period include death losses of 11.4% of cloned calves at birth, 13% within 48 h after birth, and 7.3% from 2–60 days after birth. Cloned goats, sheep, and pigs have also been reported with increased likelihood of death in the neonatal and early juvenile period. Although a majority of clones are born normal and apparently healthy, mortality rates of clones described in many reports are a major limitation of cloning technology and represent substantial economic loss as well as justifiable animal health and welfare concerns. Experiments were undertaken in our laboratory to identify the fundamental physiological differences between cloned and normal calves with the objective of developing neonatal care protocols to maximize health and well-being of cloned offspring. Clones and controls were carried in the same group of recipient females and were delivered by cesarean following induced labor. Physical evaluations and measurements of blood biochemistry, gases, and hematology were performed within 10 minutes of birth and at 1, 6, 12, 18, 24, 36 and 48 h after birth. Ultrasonographic examinations of cardiac, thoracic, umbilical, and abdominal regions were completed at 24 h following birth and at 4 weeks of age. General health conditions that were appraised documented the high-risk status of cloned neonates as all cloned calves were observed with at least one periparturient event associated with high-risk calves, and several clones were observed with six or more high-risk events. Cloned calves were more variable for many of the measures of blood biochemistry and hematology and were observed with hypoglycemia, anemia, thymolysergulation, and enlarged umbilical structures. Placentas from both clones and controls were collected by hysterectomy of the dam immediately following delivery of the calf and placentomes were counted, weighed, measured, and classified by shape. Cloned placentomes contained significantly fewer placentomes, a shift in placentome morphology from the normal mushroom-like structure to a flattened, pancake-like structure, and a two-fold increase in placentome surface area and mass. Such observations suggest that conditions in utero for cloned fetuses may be considerably different from the fetal environment of non-clone pregnancies. All cloned calves in the present study survived the 48-h neonatal period; however 50% of calves were lost during the pre-weaning period. All control calves survived to weaning and subsequently. Experimental evidence exists in several species that aberrant conditions in utero result in reduced survival of the neonate and increased susceptibility to disease in later life, a pattern that appears to be consistent with survival data currently reported in clones of all species.

Meat and milk compositions of bovine clones

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The technology is now available for commercial cloning of farm animals for food production, but is the food safe for consumers? Here, we provide data on more than 100 parameters that compare the composition of meat and milk from beef and dairy cattle derived from cloning, versus those of genetic and breed matched control animals from conventional reproduction. The cloned animals and the comparators were managed under the same conditions and received the same diet. The composition of the meat and milk from the clones were largely not statistically different from those of matched comparators, and all parameters examined were within the normal industry standards or previously reported values. The data generated from our match-controlled experiments provide new science-based information desired by regulatory agencies to address public concerns about the safety of meat and milk from somatic animal clones.

Overexpression of myostatin in fish

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Muscle growth and development in domestic animals are controlled by an array of intrinsic and extrinsic factors. One of the more recently discovered regulators is myostatin, a member of the transforming growth factor β (TGF-β) superfamily. Myostatin is synthesized in muscle cells and inhibits proliferation and differentiation of myoblasts, limiting growth rate and muscle mass in domestic animals. Disruption or inactivation of the myostatin gene has been demonstrated to markedly enhance growth in several species of domestic and laboratory animals. The most striking example of myostatin inactivation is observed in the double-muscled Belgian Blue breed of cattle, a breed that carries an 11-nucleotide deletion inactivating expression to muscle tissue. The results of these experiments provide new science-based information desired by regulatory agencies to address public concerns about the safety of meat and milk from somatic animal clones.
Cecropin transgenic catfish and studies toward commercial application

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Transgenic fish have been produced that exhibit accelerated growth rates, increased disease resistance, altered body shape and composition, altered coloration, expression of anti-freeze proteins and potential sterility. The cecropin B gene from the moth Hyalophora cecropia was transferred to the channel catfish Ictalurus punctatus. Transgenic individuals P1 were mated to produce individuals F1 that exhibited enhanced disease resistance and survival when challenged with pathogenic bacteria. During anpizootic of Flavobacterium columnare in an earthen pond, the percentage of transgenic individuals containing preprocecropin B construct that survived (100%) was significantly greater (P < 0.005) than that of nontransgenic controls (27.3%). Also, when challenged in tanks with Edwardsiella ictaluri, the causative agent of enteric septicaemia of catfish, the percentage of transgenic individuals containing cecropin B construct that survived (40.7%) was significantly greater (P < 0.01) than that of nontransgenic controls (14.8%). There were no pleiotropic effects of the transgenes, and growth rates of the transgenic and nontransgenic siblings were not different (P > 0.05). Similar results have been obtained in other species.

The primary impediment to commercialization of transgenic fish is the concern about potential environmental impacts. Environmental risk assessment to this point in time indicates that it is highly doubtful that adverse environmental impacts would occur if transgenic fish were to escape into the “natural” environment. However, despite these data, government approval of various transgenic fish will be slow, and this is the impetus for the current research on transgenic sterilization, which if successful, would eliminate almost all potential environmental impact. Constructs have been studied that have shown promise to disrupt embryonic development or gamete maturation in fish.

Is there a non-target animal safety pathway to environmental risk assessment?

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FDA regulation of transgenic animals in the U.S. has been criticized as inadequate with respect to environmental impact oversight because the Federal Food, Drug and Cosmetics Act does not contain an explicit environmental safety mandate and assessment under the National Environmental Policy Act can reveal impacts but may not be able to compel environmental safety. While this critique is controversial and is not accepted by FDA, there is no disagreement that the FFDCA does require a demonstration of safety to untreated animals that may come into contact with a transgenic animal or its metabolites. Agency guidance with respect to NEPA assessment of human drugs and biologics recognizes an ecological dimension to animal safety assessment that is broader than the simple toxicity measurement typically applied to pharmaceutical compounds and includes “lasting effects on ecological community dynamics.” Here I discuss whether a wider view of nontarget animal safety provides an additional pathway to environmental assessment, independent of but complementary to NEPA.

The FDA investigation into the structure and function of a transgenic phenotype provides a robust, evidence-based and bottom-up analysis of the treated animal and of differences in physiology or behavior from the parent species. This rich data set can be designed to fully locate an enhanced trait in relation to the pool of physiological and behavioral responses to environmental challenge across the range of variation described by natural and domesticated phenotypes within the parent species. The level of phenotypic detail generated through the FDA investigation is rarely available in conventional environmental impact assessment. Careful attention paid in the NTA analysis to the metabolic balance achieved in a transgenic animal between an enhanced functionality and the energetic cost of its deployment may provide powerful evidence of the potential inherent in a trait-enhanced phenotype for more site-specific downstream jurisdictions governing land and water use, discharge, wildlife protection and ex-US standards for transgenic animal culture.

Robust nontarget animal safety assessment can anchor environmental assessment in the predictable consequences of measurable phenotypic change, increase the clarity of hypotheses about potential impacts and simplify downstream analysis of the receiving environment at a specific site. Examples drawn from transgenic Atlantic salmon studies illustrate the discussion.

Expression of Lewis a antigen in the milk of cloned calves transferred with human alpha-1,3/4-fucosyltransferase gene

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The human Lewis(a) blood group antigen, a kind of fucosylated antigen, is one of the receptors for a number of potentially pathogenic microorganisms. Widely distributed microbial surface adhesive can bind Lewis(a) antigen, which plays a crucial role in establishing successful infection of the host for pathogenic microorganisms. Human alpha-1,3/4-fucosyltransferase catalyzes the conversion of lacto-N-tetraose (LNT) precursor to Lewis(a) antigen. The objective of the present research was to produce alpha-1,3/4-fucosyltransferase transgenic calves, which express Lewis(a) antigen in their fucosylated milk to compete the adherence of pathogenic microorganisms with Lewis(a) antigen in human gastrointestinal tract. Fetal fibroblasts were isolated from a Day-67 fetus of a Holstein cow, and were transfected with a mammary gland-specific expression construct containing the enhanced green fluorescent protein (EGFP), neomycin resistance (NEO), and human alpha-1,3/4-fucosyltransferase genes. After selection with neomycin (G418) for about 14 days, a population of G418-resistant cells was used as nuclear donors.
Increased casein gene dosage and its implications for the composition of milk

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We have previously generated transgenic cattle with additional copies of bovine β- and κ-casein genes using somatic cell nuclear transfer. The increased casein gene dosage was intended to boost the level of the milk proteins β- and κ-casein and thereby enhance milk composition and milk processing characteristics. In particular a change in κ-casein, which is thought to be located at the surface of the colloidal milk protein particles, was expected to affect the size of these so-called casein micelles and thus the physicochemical characteristics of the milk.

The milk produced by these transgenic cows after a hormonally-induced lactation showed an altered composition, most strikingly a two-fold increased κ-casein content. The first natural lactation of these cows gave access to larger quantities of milk over an extended period. In conjunction with the introduction of transgenes encoding the distinctive variants β-casein A3 and κ-casein B, this provided the opportunity for a detailed analysis of the effects of an increased casein gene dosage on milk composition. We have analyzed milk from two high-expressing transgenic lines for basic milk composition at early, peak, mid and late lactation. A distinctive change in color from white to yellow for the milk produced by the transgenic cows was a clear indication that the milk composition has been significantly altered generating a novel milk with unique processing properties. The introduction of additional β- and κ-casein genes resulted not only in the expected expression of the transgene derived proteins but led to complex changes in the expression levels of other milk proteins. Unexpectedly, it also affected two other major milk components, namely fat and minerals, whereas the level of lactose was unchanged. In summary, this novel milk presents a unique model that is likely to provide not only insights into the fundamental regulatory processes synchronizing the activities of the mammary gland but also a better understanding of the processing qualities of milk.

Agricultural biotechnology 10 years on: lessons from photosynthesizing organisms

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Since the first biotech crop was commercialized in 1996, adoption of biotech crops has expanded rapidly. In 2004, these crops were grown on 81.0 M hectares (200 M acres) by 8.25 million farmers in 17 countries, which represent a 20% growth rate over 2003. The global commercial value of biotech crops grown in 2003–2004 crop year was US$44 billion, 98% from five countries, United States (U.S.), Argentina, China, Canada and Brazil, growing one or more of biotech crops: soybeans, cotton, corn and canola.

To date, the U.S. is the leader in production, with $27.5 billion in value in 2003–2004 from soybeans, corn, cotton and canola. In the next decade some studies estimate the global value of biotech crops will increase nearly five-fold to $210 billion. Although North America leads in research, more than half of the 63 countries engaged in biotech research, development and production are developing countries. Western Europe, China, Argentina, Brazil, South Africa, Australia and India are centers of influence that will help lead development into the future, a recent study by Rung et al., found. China has emerged as a major center for biotech research. Its government has invested several hundred million dollars, ranking it second in the world in biotech research funding behind the United States. But push-back from the European Union (EU) is affecting approval and commercialization rates.

U.S. consumer attitudes also tend to be positive on the whole. It is notable that consumers do not mention products of biotechnology as avoided foods on an unaided basis. In fact, in an International Food Information Council (IFIC) survey conducted in 2004 a clear majority of consumers (59%) believed that the technology will benefit them or their families within the next 5 years. The survey also found that 80% of Americans could not think of any information "not currently included on food labels" that they would like to see added. Ten percent identified nutritional content, 4% identified ingredients, and only 1% identified biotechnology as information they would like to see added to a food label.

While biotech research and development in Europe slowed significantly following the EU’s 1998 de facto moratorium on approvals, which has since been lifted, Europe's stance on biotech crops can not prevent biotech adoption in the rest of the world. According to Runge et al., as the EU becomes increasingly isolated, it will discourage its young scientists and technicians from pursuing European careers. If, on the other hand, the EU engages biotech in an orderly regulatory framework harmonized with the rest of the world, it will encourage a more rapid international diffusion of the technology.

The future of animal biotech will be considered in this context with consideration of the potentially lucrative and controversial area where these technologies overlap, namely pharmaceutical production.
Poster Abstracts

Demonstration of RNA interference in porcine cells

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The most recent and promising technique by which genes can be silenced is RNA interference (RNAi). RNAi is activated by the presence of long double-stranded RNAs (dsRNAs) which are cleaved by the enzyme Dicer into short 21-23 bp duplexes known as short interfering RNAs (siRNAs). The antisense strand of the siRNA, which is complimentary to the target mRNA, binds to the RNA-induced silencing complex (RISC) to guide the RISC to the target mRNA. RISC then degrades the target mRNA. RNAi can be activated by introduction of artificially synthesized siRNA or by expression of short hairpin RNAs (shRNAs) using a Polymerase III promoter. Success using RNAi has been demonstrated mainly in human and mice, however this technique has not been tested in livestock, where such an approach would be valuable in studying the function of economically important genes. In this experiment, the efficiency of RNAi in silencing endogenous myostatin (GDF8) was tested in porcine cells. GDF8 is a negative regulator of skeletal muscle and the effects of its loss-of-function can be seen in double-muscle breeds of cattle where skeletal muscle mass is 20% higher than in “normal” cattle, causing the meat to be more lean and tender. It is anticipated that a similar loss-of-function of myostatin could be induced in pigs to generate the same phenotypic effects.

Five shRNA constructs were created to test whether RNAi could be used to silence endogenous myostatin. RNA was extracted from cells after 48 h and the relative expression of myostatin present in targeted and untargeted cells was evaluated by Real Time RT-PCR analysis. Results show that myostatin expression in targeted cells was decreased up to 97% when compared to untargeted cells with variation in efficiencies among the different constructs. Induction of the interferon response was also determined by examining expression of the 5'-3' oligoadenylate synthetase 1 gene (OAS1) in targeted cells compared to non-targeted cells. Some constructs demonstrated increased OAS1 mRNA levels of up to 4 times when compared to background. It is still to be determined whether the interferon response was induced. These results demonstrate that RNAi is a suitable tool for gene-specific silencing of porcine genes in vitro.

Endogenous RNAi expression: exploiting miRNAs for stable, tissue-specific gene knockdown

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RNA interference (RNAi) has revolutionized loss-of-function genetics in mammals. Transient knockdown of targeted transcripts is possible with synthetic short-interfering RNA (siRNA), or Stealth™ molecules. Alternatively, the expression of related RNA structures from intracellular interfering RNA (siRNA) molecules can achieve stable knockdown in cells and transgenic animals. To date, the most common intracellular knockdown technologies use polIII promoters (such as U6 and H1) to drive expression of short-hairpin RNA (shRNA). Here, we report on the construction of Pol II-driven miRNA-based RNAi vectors with functionality superior to traditional shRNA. The vectors express artificial miRNAs designed to perfectly complement, and therefore cleave, endogenous target transcripts with a high rate of success. Regulated and tissue-specific patterns of expression from Pol II promoters have been investigated in labs around the world; with this system, select Pol II promoters are readily interchangeable using Gateway® recombination. Emerald GFP is also encoded in the same transcript as the miRNA to reliably report expression, and sites are included to facilitate cloning multiple miRNAs in tandem to simultaneously target more than one gene, or more than one site in a single gene. Examples targeting endogenous genes and delivery of the system with lentivirus will be presented.

Knockdown of bovine prion expression by RNA interference

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RNA interference (RNAi) is a recently developed alternative to gene knockout technology for ablation of gene expression. Neurons in which PrP expression was knocked down (KD) by introduction of short, interfering RNA molecules (siRNA) were transiently resistant to infection by PrP-scrapie (Daude et al., J Cell Sci 116: 2775, 2003). Thus, RNAi may serve as means of conferring resistance to prion disease. To KD PrP expression in the bovine, fibroblasts were transfected with a pluper siRNA vector designed to express a neo selectable marker and siRNA targeted to a sequence within the PrP coding region. A control vector expressing a scrambled version of the PrP sequence was also generated. Cells were transfected with either PrP-specific or control vectors and selected with 800 ug/ml G418 for 14 days. G418-resistant clones were expanded through 22 cell doublings. Five clones from each treatment were selected for further analysis. Mean cell doubling time of clones was 35.36 h and did not differ (P>0.05) between PrP-specific siRNA and control transfectants. Clones were analyzed for PrP expression by quantitative PCR (QPCR) and western blot. For QPCR, cDNA derived from PrP mRNA was detected on the ABI7700 system, using primers and TaqMan probes. PrP mRNA abundance in each sample was normalized internally to the 18S rRNA subunit. PrP mRNA expression was reduced (P<0.05) by 68.43%. Prion protein was detected on western blots using a polyclonal anti-bovine PrP antibody and chemiluminescence. Exposure densities from PrP-specific siRNA and control clones were compared using NIH Image software. PrP expression was reduced (P<0.05) by 75.25% in 4/5 clones.
transfected with PrP-specific siRNA vs. control. RNAi was thus capable of knocking down PrP expression in bovine fibroblasts. We are using PrP KD cell lines to generate PrP KD cattle by nuclear transfer.

Evaluation of dual site-specific recombination systems in Danio rerio for the biological containment of transgenic fish

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Unless total physical containment is obtainable, the transgenic manipulation of fish will likely depend upon the development of biocontainment approaches to prevent the undesired spread of genetically engineered DNA into natural populations. Germline-specific expression of a self-excising recombinase in genetically engineered plants and animals has the potential to remove both the target DNA and the recombinase DNA and minimize the ecological risks of transgene flow from genetically engineered organisms. The use of site-specific recombinases has been extensively documented in a wide array of organisms, including plants, flies, and mice. Recent research describes the first use of a site-specific recombinase, Cre/loxP, in zebrafish. Most studies involving site-specific recombination employ the use of only one type of recombinase. When used together, Cre and FLPe recombinases offer more sophisticated genetic manipulations than can be achieved by employing either site-specific recombinase alone. We plan to investigate the use of zebrafish as a teleost model to evaluate the tandem use of two recombinase systems, Cre/loxP and FLPe/FRT, to excise transgenes and their regulatory elements from the germline of otherwise fertile transgenic fish. Our approach is to remove the transgene from the sperm and eggs while still retaining the advantageous effects of the transgene in the somatic tissues of the fish. Our first objective was to develop a construct capable of directing germline-specific expression. We built a vector containing the zebrafish vasat promoter and 3′ UTR driving the expression of a fluorescent marker gene, DsRed-Express. This construct was microinjected into zebrafish embryos, and gamete-specific expression of the marker gene in transgenic founder fish was subsequently (~2.5 months) observed. When they reached sexual maturity, both male and female transgenic founder fish were mated to wild-type fish to confirm the zygotic inheritance and activity of both the maternally- and paternally-inherited transgene. Our second objective was to build recombinase/fluorescent translational fusion proteins to aid in tracking the activity of the recombinases. We assured the dual functionality of these fusion proteins in Schizosaccharomyces pombe. We present here our current progress toward completing these two first objectives.

Concerns regarding the use of viral DNA sequences in transgenesis and gene therapy

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A literature search revealed that viral delivery systems are used in multiple food and companion animal species for a variety of purposes. Some examples include retroviral transfection of poultry to produce human pharmaceuticals in eggs, expression of marker genes in swine, and gene therapy in cats and dogs. In addition, recombinant DNA constructs can contain sequences from various viruses, including regulatory or coding regions. The most commonly used viral DNA sequences are those from retroviruses and adenoviruses. Less commonly used sequences are from paroviruses, polyomaviruses, poxviruses, arterivirus-es, dicistroviruses, flaviviruses, and coronaviruses. Currently, this type of research is conducted in laboratory settings under tight controls. However, the potential uses of this technology in food-producing and companion animal species are quite broad. Therefore, one should consider the potential hazards of vector technology and risk-mitigation options. An identified hazard associated with this technology includes the recombination of viral sequences with wild-type viruses. This poses the potential risk of host range expansion, enhanced virulence, and tumorigenesis. Animals at risk for these adverse outcomes include target animals, non-target individuals of the same species, other non-target domestic species, humans (consumers, pet owners, animal handlers), and other animals in the environment (fish, birds, mammals, insects, etc.). The risks associated with use of viral DNA may be reduced by careful vector design, the use of self-inactivating viruses, helper-dependent viruses, virus-free amplification systems, or avoidance of viral DNA sequence use in the vectors.

Probabilistic risk assessment of transgene invasion

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Environmentalists remain wary of the safety of transgene (TG) technology. The first step to increase confidence in transgenic products and alleviate concerns is to develop a risk assessment methodology agreed upon by scientists. This goal can best be accomplished using a thorough, unbiased examination of the associated risks and hazards. Muir and Howard developed a model for predicting invasion risk of transgenes introduced into wild populations by escaped genetically modified organisms (GMO). Transgene fate can be completely determined by six major life history characteristics (net fitness components) common to all species. As these components can be measured in secure settings, this method could be utilized by regulators to evaluate the environmental risk of any transgenic diploid organism. In its current form, however, the model is deterministic, i.e., random events and uncertainty in fitness component estimation are not reflected in model predictions. Here we propose a Monte Carlo implementation of Muir and Howard’s fitness component model using Zebrafish (Brachydanio rerio) as a model organism. Fitness components will be estimated, using both linear models and non-parametric bootstrap methods, and
entered into the model to predict transgene fate. Predictions of transgene fate in wild populations will be expressed as probability statements rather than point estimates. Model predictions will be tested and validated using replicated fish populations in secure, simple ecosystems. This research will enhance methods of examining risks and hazards associated with agricultural biotechnology, and will give regulatory agencies an important statistical decision-making tool.

**Lentiviral vector transgenesis of the chick: applications to developmental studies**

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Studies of the chick embryo have made very significant contributions to understanding the development of vertebrate embryos. As the chicken genome is now available, the chick model system has become even more useful and the availability of a robust method for transgenesis of the chick will be an essential tool for exploiting the system. Transgenesis can be used directly to analyse the control of expression of genes involved in development or to investigate their function in development. We have shown that lentiviral vectors derived from the lentivirus equine infectious anaemia virus (EIAV) can be used to generate transgenic birds with high efficiency.

To establish if tissue-specific gene expression can be achieved using transgenes introduced using an EIAV vector, we generated transgenic birds using a vector with the regulatory sequence of the rat MLC gene linked to lacZ reporter gene. Seven founder transgenic males were generated; 70% of founder males hatched. These were mated to generate eight G1 hemizygous transgenic birds, and these were subsequently analysed for lacZ expression. Expression was limited to the myotome of developing embryos and to adult skeletal muscle.

A valuable tool for studying lineage in developing embryos has been the use of quail/chick grafts where cells derived from the quail can be identified by staining fixed material after fixation and embedding in paraffin wax. The graft experiments have showed that the quail cells cannot be followed during development until the experiment is terminated.

We have established transgenic birds carrying a ubiquitous reporter gene, using the CAG (CMV-chicken β-actin) enhancer/promoter driving expression of green fluorescent protein (GFP). GFP expression is visible in hemizygous G1 embryos in the new-laid egg and throughout subsequent development. The utility of these embryos in grafts into wild-type embryos has been demonstrated at early stages of development, growing embryos in new culture, and at later stages using windowed eggs to access the recipient embryo.

**Construction of chicken oviduct-specific gene targeting vector and preparation of transgenic DT40 cell lines**

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To produce transgenic chickens, three vectors were constructed. Two of them are oviduct-specific gene targeting vector, which contain chicken ovalbumin 5′ regulatory region and GFP gene. The foreign gene can be integrated into the genome of chicken and expressed under the regulation of chicken ovalbumin 5′ regulatory region. Another secretory fusion vector phLepGFP-secretion was constructed, which contains human obese gene and GFP gene. The foreign gene can be co-expressed and secreted leading by lysozyme signal sequence. The vector phLepGFP-secretion was transfected into the chicken embryo fibroblast, and green fluorescent light was observed. This indicated that transgene was expressed successfully. After that, the vector was transfected into chicken bursal lymphoma cell line (DT40) to obtain the transgenic cell line with the presence of G418. Forty-four of 52 cell clones were confirmed to be positive by PCR, which meant transgene were integrated into the genome of DT40 cells. The cell culture supernates were detected by ELISA to explore if human leptin existed. The results showed that human leptin was secreted to the cell culture supernates, and the concentration of leptin was up to 512 pg/ml per 10^6 cells. These gene-modified cells can be injected into hens, and the protein secreted by cells can be gained from the eggs laid by the hens.

**Lentiviral vector transgenesis of the chick: exogenous protein expression in the oviduct of transgenic hens**

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We have shown that vectors derived from the lentivirus equine infectious anaemia virus (EIAV) can be used to generate transgenic chickens with high efficiency. One of the potential applications of avian transgenesis is the production of birds that synthesize therapeutic proteins that can then be purified from laid eggs. This has been proposed to be a useful alternative to production of proteins in large-scale mammalian cell culture or in the mammary glands of transgenic mammals. The potential advantages of pharmaceutical protein production in laying hens include lower costs, high egg-laying capacity and appropriate glycosylation.

Egg white is produced in the oviduct of mature hens and is composed of four major proteins, of which ovalbumin is the most abundant (approximately 2.2 g per egg, 55% of total egg white protein). The sequences involved in regulation of expression of the ovalbumin gene have been partially characterized by transfection of oviduct cells in culture, but the regulatory sequences require analysis in transgenic hens for full characterization. We have taken sequences of different lengths from the 5′ end of the ovalbumin gene, linked these with the reporter gene GFP and cloned them into an EIAV vector. Packaged viral vector preparations were injected into chick embryos in new laid eggs and the embryos cultured to hatch. We analyzed G0 chimeric hens for expression of GFP in the oviduct when they came into lay. Expression was detected in patches of the oviduct of several hens. This analysis indicates that the longer ovalbumin 5′ region gave a higher level of GFP expression and suggests that expression using this sequence is tissue-specific.
Microbiological properties of cheese made from human lysozyme transgenic goat milk

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Genetically engineered dairy goats expressing elevated levels of the bacteriostatic enzyme lysozyme in their milk were developed with the goal of maintaining udder health, increasing product shelf-life and improving consumer health. The purpose of this study was to evaluate the effect of the production of lysozyme in milk on the desirable lactic acid bacteria (LAB) during cheese-making. While the growth of contaminating bacteria is a concern in terms of consumer health and product quality, the ripening and development of the sensory characteristics of cheese is associated with changes in the LAB profile. The increased levels of lysozyme in the milk of the transgenic animals would therefore be expected to significantly alter typical LAB profiles; however, it is unclear whether the net effect would be beneficial or detrimental.

Milk from lactating transgenic goats and age, parity and stage of lactation-matched non-transgenic controls was transformed into cheeses using standard industry methods with a commercially available LAB starter culture and their microbiological load evaluated throughout production. Analysis was performed on initial milk samples and from cheeses on days 4 and 9 of production. After dilution (milk) or homogenization followed by dilution (cheeses) in sterile sodium citrate, samples were plated on square plates by track dilution and incubated at 37°C for 48 h to allow for colony formation. Resulting colonies were then counted using imaging software. Two different media were used to quantify the numbers of lactococci (M17 agar) and lactobacilli (MRS agar) present in each sample. The production of lysozyme in milk affected the growth of both LAB populations. On day 4 of cheese production, the mean number of colony forming units (cfu) of lactococci was significantly lower (P < 0.05) in transgenic samples than controls. Mean numbers of lactobacilli were observed to significantly decrease (P < 0.02) between days 4 and 9 in control but not transgenic samples, whereas the mean numbers of lactococci significantly increased (P < 0.02) in transgenic samples during the same period.

Two populations of LAB generally play a role in the transformation of milk into cheese. Starter LAB reduce the pH resulting in curd formation then die off and a second wave of LAB develop and contribute to the flavor, texture and ripening of the cheese. Our results indicate that lysozyme is affecting both of these populations of LAB. The consequences of the lysozyme-induced alteration of the LAB profile during cheese-making are being further investigated.

Brain fatty acid composition and postnatal growth of neonates raised on transgenic milk high in omega-3 fatty acids

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Omega-3 (n3) polyunsaturated fatty acids (PUFA), especially the longer-chain docosahexaenoic acid (22:6n3, DHA), have important functional and structural effects on neonatal and infant development. DHA is incorporated into the phospholipid membranes of the brain and retina during intrauterine development and continues to amass through the age of 2 years. DHA is currently absent from most infant formulas marketed in North America, and although present in breast milk, the proportion of n3 PUFAs has been observed to be decreasing over time as a result of the reduced consumption of foods rich in n3 PUFAs in the Western diet. It has been found that brain, plasma, and erythrocyte phospholipid levels of DHA are lower in formula-fed versus breast-fed infants, suggesting that formula-fed infants may not synthesize enough DHA from alpha-linolenic acid (ALA, 18:3n3) to permit deposition in the brain at a rate equivalent to that of breast-fed infants. The nematode Caenorhabditis elegans is able to synthesize ALA by virtue of an endogenous omega-3 fatty acid desaturase that recognizes a range of 18- and 20-carbon n6 substrates. We generated transgenic mice expressing the C. elegans omega-3 desaturase under the control of a lactation-induced mammary gland promoter. Milk from these transgenic mice had significantly increased levels of n3 PUFA (ALA and eicosapentaenoic acid EPA, 20:5n3). These increases were most pronounced in the phospholipid fraction of the transgenic mouse milk (http://www.intlpag.org/pag13/abstracts/PAG13_P763.html). To examine the postnatal effects of consuming the omega-3 enriched milk we compared the weights, and the brain and plasma fatty acid composition of mouse pups nursing on transgenic dams to those observed for pups raised on the milk from nontransgenic dams. Transgenic and nontransgenic full sib females were placed on a diet containing 11% fat composed primarily of safflower oil (78% linoleic acid, 18:2n6) at weaning. Milk samples were collected at 10 and 12 days subsequent to parturition. Pups were weighed at 10 days of age and again at weaning (21 days), at which time they were sacrificed to allow for the collection of brain and blood plasma samples. Fatty acid profiles of the milk, pup blood and brains were analyzed by gas chromatography. Results will be discussed.

Cloning and characterization of lysophosphatidic acid acyltransferase (LPAAT) from the Echidna (Tachyglossus aculeatus)

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The structure of milk fat triacylglycerides (TAGs) has important human health implications. The absorption of dietary fatty acids in the sn-2 position is favored over fatty acids in the sn-1 and sn-3 positions. From a human health perspective it would be desirable to decrease the consumption of fats containing saturated fatty acids in the sn-2 position. The fatty acid distribution in TAGs in the milk fat of most mammals consists of saturated fatty acids in the sn-2 position. However, the echidna (Tachyglossus aculeatus), a primitive monotreme mammal of New Guinea and Australia, produces milk fat TAGs with
Development of methods for the production of transgenic quail expressing an *E. coli* phytase gene

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Excess phosphorus (P) in manure is a major environmental pollutant associated with poultry and swine production and is directly related to eutrophication, which results in the death of aquatic organisms and loss of biodiversity. Phytate accounts for 50–80% of plant phosphorus and this phytate-bound P is unavailable to simple-stomached animals. As a result, this P is not utilized by the animal and is therefore excreted. Phytases are enzymes that cleave inorganic phosphate from phytic acid, thereby improving its bioavailability to simple-stomached animals. Strategies intended to limit levels of P excretion in production animals have included dietary supplementation of phytase, as well as the use of transgenic crops with reduced phytate levels. Additionally, the recent production of transgenic pigs that express recombinant phytase in the digestive tract has been shown to increase phytate-P availability and therefore reduce P excretion. We have developed a phytase transgene construct for production of transgenic quail as a model system for application to commercial poultry. We have isolated and cloned the chicken proprostaglin promoter (PPGP), which is designed to limit the expression of a recombinant phytase to the proventriculus where it will be secreted into the upper GI tract. The PPGP was cloned from genomic DNA isolated from the Cal Poly chicken population, and the phytase gene was cloned from an *E. coli* isolated from the Cal Poly quail population. The phytase gene sequence is distinct from other *AppA* phytases previously reported, and codes for a functional phytase enzyme. Expression of this new phytase in a yeast system has yielded a functional protein with enzymatic properties highly suited for use in a recombinant poultry system. We have developed a protocol for the isolation and culture of quail primordial germ cells (PGC) from 5.5-day-old embryos for transfection and repopulation of a developing embryo with PGCs harboring the transgene construct. A green fluorescent protein gene controlled by the T7 promoter was included in the construct to allow for the simple assessment of transgene incorporation in cells, developing embryos, and tissues of the adult animals.

**Differential expression of proteins in the parotid gland of the Enviropig™**

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When considering the molecular profile of a transgenic organism, especially those that are inherently complex such as livestock, potentially many expression pathways can be altered due to the insertion and expression of the transgene. Such effects could range from a disruption of neighboring genes to an overall up-regulation of harmful proteins or metabolites. In order to survey these changes, researchers in recent years have turned to genomic (microarrays or SAGE) and proteomic (2-D gel electrophoresis, or isotope coded affinity tagging) profiling technologies. These molecular profiling techniques have the potential of enabling the wide-range detection of unintended changes that otherwise would be exceedingly difficult to detect.

The Enviropig™ is a breed of transgenic pigs developed at the University of Guelph that secrete an enzyme called phytase in their saliva resulting in a 60-75% decrease in environmental fecal phosphorus pollution. In this study isotope tagging for relative and absolute quantification (iTRAQ) was used to detect changes that may have occurred in the proteome of the Enviropig parotid gland due to the insertion and expression of the phytase transgene. Analysis of tissue from the parotid gland of the Cassie line of the Enviropig™ breed and conventional Yorkshire pigs identified 212 common proteins at a confidence cutoff of >99%. Among the proteins identified was phytase, which was shown to be greatly over-expressed in the Cassie line. Three proteins identified by iTRAQ analysis to be down-regulated in the Enviropig (saliary lipocalin precursor (SAL), α-amylase (AMY), and lysozyme C-1 (LYS)), and the endogenous parotid secretary protein (PSP, not detected by iTRAQ but hypothesized to be down-regulated) were selected for differential expression analysis with greater replication by real-time RT-PCR. Expression of SAL, AMY, LYS and PSP was down-regulated by a fold-change of 1.71 (*P* = 0.23), 3.53 (*P* = 0.03), 2.01 (*P* = 0.03) and 1.80 (*P* = 0.23), respectively.
Despite the decrease in the expression of these genes, the Cassie line performs similar to conventional Yorkshire pigs. These techniques are currently being applied to primary food tissues.

Full-term development of a cloned rabbit by somatic cell nuclear transfer

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Transgenic/knockout (KO) rabbits can serve as an excellent animal model for human cardiovascular diseases (CVD) and other diseases. However, the production of transgenic/KO rabbits is hindered by low efficiency of traditional DNA microinjection and the unavailability of embryonic stem cell lines. An alternative approach is to produce transgenic/KO rabbits by somatic cell nuclear transfer (SCNT) using genetically modified somatic cells as nuclear donors. Our initial objective of the study was to prove the feasibility of cloning rabbits by SCNT, since rabbit was a difficult species to be cloned. Rabbit oocytes were flushed from the oviducts of superovulated donors treated with the regime of FSH and hCG. Cumulus cells were then denuded from the oocytes by incubation in 0.5% hyaluronidase and pipetting. Oocyte enucleation was conducted in 10% FBS M199 and confirmed by fluorescent microscopy. Cumulus cells used for nuclear donors were prepared from fresh cumulus-oocytes complexes. Donor nucleus was transferred into a recipient oocyte by either cell fusion or direct nuclear injection method. In the cell fusion method, a small donor cell with the diameter approximately 15-19 μm was transferred into the perivitelline space of an enucleated oocyte; subsequently the somatic cell-cytoplast pair was fused by applying three direct current pulses at 3.2 kV/cm to produce a fusion or direct nuclear injection method. In the cell fusion method, a mechanically lysed donor cell was injected into oocyte cytoplasm with the aid of Piezo-Drill system. Fused embryos or injected oocytes were activated by the same electrical stimulation regime described above, and subsequently cultured in M199+10% FBS containing 2.0 mM 6-dimethylaminopurine (DMAP), 5 μg/ml cycloheximide for 2 h. For in vitro study, cloned embryos were cultured in B2 medium plus 2.5% FBS for 5 days (initiation of activation=day 0) at 38.5 °C in 5% CO2 humidified air. For in vitro study, cloned embryos were cultured for 20-22 h in vitro before transfer into pseudopregnant rabbit recipients. Pregnancy was monitored by palpation and/or ultrasound on Day 14-16 post embryo transfer (ET).

The results (Table 1) showed that the donor nuclei introducing rate was higher with nuclear direct injection than cell fusion method ($P<0.05$). There was no significant difference among subsequent cleavage and development to morula and blastocysts between both methods, though the development rates of cloned embryos via electrical mediated fusion were higher than those derived from injection group. One recipient in the injection group (1/6, 17%), and six recipients in the fusion group (6/16, 38%) were diagnosed as pregnant. A full-term cloned rabbit from the fusion group was delivered on Day 33 post ET. The cloned rabbit was a stillborn, but showed a normal morphology. To our knowledge, this is the second report of full term development of cloned rabbit by somatic nuclear transfer cloning. Our further study is to clone live rabbit offspring with modified transgenic/KO somatic cell lines (Supported by NIH/NCRR-SBIR grant: 1R43RR020261-01).

Synchronization and superovulation of cycling gilts: a window of opportunity

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We have developed an efficient protocol to synchronize and superovulate a population of mature pigs for the collection of pronuclear stage embryos. A timed and coordinated regimen of Lutalyse®, P.G.600® and Chorulon® along with daily heat checking allowed synchronization of groups of cycling gilts with a cycle variation of 7 days. Cycling pigs in the range of 10–16 days after their previous start of standing estrus have been successfully synchronized using this protocol. There is no need to use alternate strategies such as the use of Regumate®. Our best protocol (Protocol4) averaged 35.8 oocytes or embryos recovered per pig with a range of 3–83. The protocol has been demonstrated to reliably produce pronuclear stage embryos for DNA microinjection.

Porcine blastocysts produced by hand-made cloning with a combined electrical and chemical activation

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Table 1. In vitro development of rabbit cloned embryos with cumulus cells as nuclear donors

| Treatment | Donor cell type | Number | No. (%) Nuclei introduced | No. (%) Cleavage | No. (%) 4-Celled | No. (%) Morula | No. (%) Blastocysts |
|-----------|----------------|--------|--------------------------|-----------------|-----------------|----------------|-------------------|
| Fusion    | Cumulus        | 44     | 23 (52)                  | 9 (39)          | 8 (35)          | 8 (35)         | 4 (17)            |
| Injection | Cumulus        | 51     | 48 (94)                  | 12 (25)         | 7 (15)          | 5 (10)         | 3 (6)             |
The purpose of our work was to establish an efficient protocol for activation of porcine nuclear transfer (NT) embryos produced by the hand-made cloning (HMC) technique. First, we investigated a combined electrical and chemical activation protocol for parthenogenetic development of in vitro matured zona-free oocytes. Oocytes were activated by a single DC pulse and subsequently cultured in cytochalasin B (CB) and cycloheximide (CHX). Developmental rates of blastocysts from activated oocytes were on average (mean±s.e.m.) 49±1% and 40±2%, respectively. Second, the activation protocol was applied in the HMC technique. Zona-free porcine oocytes were bisected and halves containing no chromatin, i.e., the cytoplasts, were selected. Reconstructed embryos were produced by a two-step fusion procedure. First, one cytoplast was fused to one fibroblast by a single pulse of 1.25 kV/cm for 80 μs, and after 1 h, the cytoplast–fibroblast pair and another cytoplast were fused and activated simultaneously by a single pulse of 0.85 kV/cm for 80 μs, and subsequently cultured in CB and CHX. The development of reconstructed embryos to the blastocyst stage was on average 21±4%. Thus, a combined electrical and chemical activation procedure resulted in efficient blastocyst development in the HMC technique.

Down and out with porcine CFTR; towards a large animal model of cystic fibrosis

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Cystic fibrosis (CF) is the most common life-shortening disease in Caucasians, affecting between 1 in 2000 and 1 in 4500 individuals. The gene involved in CF, the transmembrane conductance regulator (CFTR), was identified in 1989. Although mouse models of CF have been generated which manifest some of the electrophysiologic characteristics of CF, their benign pulmonary phenotype renders these models somewhat irrelevant. We have undertaken the development of a porcine model of CF.

A two pronged approach has been adopted for ablation of porcine CFTR function; homologous recombination to introduce the *F508 mutation, and RNAi of porcine CFTR using the Sleeping Beauty (SB) Transposon system. Our cellular resources include traditional porcine fetal fibroblasts (PFF) and pig multipotent adult progenitor cells (pMAPC). pMAPCs may be ideal for the extended culture required for double selection for homologous recombination, as they can proliferate extensively in culture (> 130 PDs) without obvious senescence or loss of differentiation potential.

We have used BAC recombineering to construct a series of replacement vectors harboring the *F508 mutation in combination with both positive and negative selection cassettes for homologous recombination. For RNAi, shRNA-expressing transposons have been developed and tested in a porcine cell line that expresses CFTR. CFTR mRNA knockdown (90–94%) was demonstrated by quantitative PCR, in agreement with an equivalent loss of function based on apical membrane Cl- current. The status and characterization of PFFs and pMAPCs transfected with constructs for both approaches will be presented, along with plans for developing a pig genetic model by nuclear transfer.