Hot Topics

- Task: Answer the Questions
  - Who Are We?
  - What Do We Do?
We Are:

- The Reproductive Sciences Laboratory
- Department of Veterinary Physiology & Pharmacology, Texas A&M University
- Texas A&M AgriLife Research
- Interdisciplinary Faculty of Reproductive Biology
- Interdisciplinary Faculty of Genetics
- Department of Animal Sciences
Approx. 100 M.S. and Ph.D. Students
Numerous Veterinary and Undergraduate Students
Approx. 300 Adult Education Students
What Do We Do?

“Animal Embryo Transfer: Cloning and Genetic Engineering”
Embryo Transfer

Movement of preimplantation embryos from the reproductive tract of the genetic mother (donor) to the reproductive tract of the surrogate mother (recipient).
Preimplantation Embryos

Zygote

2-Celled

4-Celled

Zona Pellucida

Pronuclei
Objectives

- To increase the number of offspring from genetically valuable females.

- The end point of most other reproductive technologies such as:
  - In-Vitro Fertilization
  - Cloning
# First Records of Successful Egg Transfer

<table>
<thead>
<tr>
<th>Date</th>
<th>Species</th>
<th>Date</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1891</td>
<td>rabbit</td>
<td>1968</td>
<td>ferret</td>
</tr>
<tr>
<td>1932</td>
<td>goat</td>
<td>1970</td>
<td>tammar wallaby</td>
</tr>
<tr>
<td>1933</td>
<td>rat</td>
<td>1974</td>
<td>horse</td>
</tr>
<tr>
<td>1933</td>
<td>sheep</td>
<td>1975</td>
<td>mink</td>
</tr>
<tr>
<td>1942</td>
<td>mouse</td>
<td>1976</td>
<td>baboon</td>
</tr>
<tr>
<td>1949</td>
<td>goat</td>
<td>1977</td>
<td>rhesus monkey</td>
</tr>
<tr>
<td>1951</td>
<td>cow</td>
<td>1978</td>
<td>man</td>
</tr>
<tr>
<td>1951</td>
<td>pig</td>
<td>1978</td>
<td>cat</td>
</tr>
<tr>
<td>1963</td>
<td>quokka</td>
<td>1979</td>
<td>dog</td>
</tr>
<tr>
<td>1964</td>
<td>golden hamster</td>
<td>1981</td>
<td>mongoban gerbil</td>
</tr>
</tbody>
</table>
First Embryo Transfer in a Livestock Species

- Warwick, B.L., R.O. Berry and W.R. Horlacher, 1932-1933

- Texas Agricultural Experiment Station

- Goats and Sheep
Calf with two mothers
First Purebred Embryo Transfer Calves Produced by a Commercial Company

- Born May 18, 1972
- Two Simmental Heifers
- Livestock Breeders International, Inc.
Granada Genetics
The Procedure
Superovulation
Insemination

2-3 times during heat period
Collection of Embryos
Location, Grading and Storage of Embryos
Cryopreservation

- Direct Transfer
  - Ethylene Glycol
  - Propylene Glycol

Voelkel & Hu, 1992
Synchronization of Recipients
Deposition of Embryos into Recipient Reproductive Tract
Sheep and Goats
In-Vitro Fertilization
Horses
The First E T Horse in the United States
Deer
Deer Embryos
Antelope
First Primate E T Offspring
1976
First Feline Embryo Transfer
1978
Dogs
“Ask not only what Nature can do for you, but also what you can do for Nature.”

— D.C. Kraemer
Reproductive Sciences Laboratory

- Dept. VTPP, Reproductive Sciences Complex, Highway 47
  - Approximately 3200 sq feet lab space (1/2 lab), 3200 sq feet office space, ots of equipment. Animal facilities
- Principal Investigators
  - Mark Westhusin, Duane Kraemer, Charles Long, Michael Golding
- Grad Students, Postdocs and Staff
  - 12 - 15
- Focus
  - Basic vs applied research, many different animal models
- Evolution
  - Embryo transfer -> in vitro embryo production -> animal cloning -> animal cloning and genetic engineering.
- History
  - ET – first cat, dog, non-human primate, white-tailed deer, other exotics, application in many other species (DCK).
  - Cloning – cattle, goats, first cat, first white tailed deer.
Current Projects at RSL

- **Cloning**
  - Most efforts focused on using for producing transgenics
  - Basic research to decipher developmental failure.

- **Development and applications of genetic engineering and RNA interference**

- **Characterization of epigenetic control of gene expression during early development**

- **Development of new methods for contraception in animals.**
Nuclear Transplantation With Embryonic Cells
Micromanipulation
Micromanipulation
Enucleation
Recombination
Electrofusion
2-cell Cloned Dog Embryo
Better Farm Animals Duplicated by Cloning

BY KEITH SCHNEIDER

WASHINGTON, Feb. 16 — A new and powerful biotechnological technology that enables livestock breeders to clone large numbers of identical animals from a single embryo is nearing commercial application in the United States and Canada.

The cloning technique is the latest in a series of breeding technologies that have allowed animal scientists to steadily increase the productivity of livestock — cattle, sheep and pigs.

Cloning, however, was a reliable technique for precisely duplicating superior animals. With the cloning technology, scientists are seeking to replicate what has long been the ultimate objective of modern horticulture: achieving the same levels of uniformity in plants and production in farm animals that were once thought to be confined only to manufactured goods.

Nowhere is the shift clear as in the use of cloning to create embryos that can be inserted into the uterus of a surrogate mother. The success of animal cloning has raised ethical concerns, but it has also ushered in a new era of animal reproduction technology.

Moreover, the ability to successfully clone larger numbers of animals raises the possibility of using the technology to produce numerous genetically identical animals. While scientists have long struggled to level the production of farm animals, they have long been unable to produce identical offspring. The cloning technology promises to make this possible.

Cloning technology has been used successfully to produce identical sheep, pigs and cattle. In the United States, cloning is now being used to produce identical animals, and in Canada, it is being used to produce identical embryos.

Cloning technology has also been used to produce identical embryos, but it has not yet been used to produce identical animals. The technology is still in its infancy, and it is not yet clear how it will be used in the future.

The cloning technology has the potential to revolutionize the way we think about animal reproduction. It is a new era of animal reproduction technology that promises to make it possible to produce identical animals.

Continued on Page 35, Col. 2.
Along Came Dolly

- Viable offspring derived from fetal and adult mammalian cells


Ten years of clones. (Top to bottom) Wolf, muflon, African wild cat, dog, sheep, mule, domestic cat, buffalo, mouse, goat, rabbit, horse, gaur, cow, pig, rat, ferret.

Jose Cibelli A Decade of Cloning Mystique Science 18 May 2007: Vol. 316. no. 5827, pp. 990 - 992
Chance

Adult male cell donor
Second Chance
Second Chance 2 Years Old
$86^2$
$86^2$
...GREAT NEWS! THEY'VE FINALLY FIGURED OUT HOW TO CLONE CATS !!!
CLONING CLINIC? NOW YOU'VE GONE TOO FAR!

*APologies to Jim Davis and Garfield*
GM Farm Animals

When Can I Order Green Eggs and Ham
Transgenic Animals

- **Increased productivity**
  - growth, feed efficiency, milk, etc
- **Disease resistance**
- **Novel products**
  - pharmaceuticals, nutriceuticals, etc
- **Xenotransplantation**
Methods for Genetic Engineering

- Pronuclear injection
- Virus mediated transfer
- Embryonic stem cells → chimeras or cloning
- Electroporation
- Liposome injection
- Sperm mediated transfer
Embryo removed from adult animal
In the laboratory, new genetic material is introduced into the embryo.
Embryo with new genetic material transferred into adult animal
Transgenic offspring express desired gene

Herds of genetically modified animals are created using natural breeding
Transgenic Mice
Transgenic pigs
Transgenic Pork Chops
Production of Pharmaceuticals in Transgenic Sheep

HIGH LEVEL EXPRESSION OF ACTIVE HUMAN ALPHA-1-ANTITRYSINS IN THE MILK OF TRANSGENIC SHEEP

G. Wright¹, A. Carver¹, D. Cottom, D. Reeves, A. Scott, P. Simons², I. Wilmut², I. Garner, and A. Colman*

Pharmaceutical Proteins Limited, Kings Buildings, West Mains Road, Edinburgh, EH9 3JQ, United Kingdom. ¹The order of these authors is arbitrary. ²AFRC Institute of Animal Physiology and Genetics Research, Edinburgh, United Kingdom. *To whom reprint requests should be addressed.

EXPRESSION OF HUMAN ANTI-HEMOPHILIC FACTOR IX IN THE MILK OF TRANSGENIC SHEEP

A. J. Clark*, H. Bessos¹, J. O. Bishop², P. Brown, S. Harris, R. Lathe³, M. McClanaghan, C. Prowse¹, J. P. Simons, C. B. A. Whitelaw and I. Wilmut

AFRC Institute of Animal Physiology and Genetics Research, Edinburgh, United Kingdom. ¹Scottish National Blood Transfusion Service, Royal Infirmary Edinburgh, EH3 9HB, Scotland. ²Department of Genetics, University of Edinburgh, West Mains Road, Edinburgh EH9 3JN, Scotland. ³LGME-CNRS & U184-INSERM, 11 Rue Humann, 67085 Strasbourg Cedex, France. *Corresponding author.
### Why is there a Transgenic Animal Bioreactor Industry?

<table>
<thead>
<tr>
<th>Drug</th>
<th>Quantity needed (Kg)</th>
<th>Cost per gram ($)</th>
<th>Market value* ($ millions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor-VIII</td>
<td>0.3</td>
<td>2,900,000</td>
<td>0.87</td>
</tr>
<tr>
<td>Factor-IX</td>
<td>4</td>
<td>40,000</td>
<td>160</td>
</tr>
<tr>
<td>GC</td>
<td>10</td>
<td>100,000</td>
<td>1,000</td>
</tr>
<tr>
<td>Protein C</td>
<td>10</td>
<td>10,0000</td>
<td>100</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>21</td>
<td>7,000</td>
<td>150</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>150</td>
<td>1,000</td>
<td>150</td>
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<tr>
<td>Albumin (hSA)</td>
<td>315,000</td>
<td>3.56</td>
<td>1,120</td>
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*US market value in USD

Total = $2.7 Billion
Mammary Gland Advantages

- Major function is to secrete protein
- Easily obtained, inexpensive and renewable
- Not harmful to the animal
- Post translational modifications: 
  - glycosylation, gamma carboxylation
- High concentration of cells - 100 to 1,000 fold higher than tissue culture
- Inducible system
- Major proteins only found in milk 40mg/ml
Financial Advantage of Genetically Modified Animals

$600 million to build/operate

This protein can be produced at either of these facilities in the same amounts. It represents a $200 million/year product in the pharmaceutical industry
THERIOGENOLOGY

PRODUCTION OF TRANSGENIC CATTLE BY PRONUCLEAR INJECTION

Granada BioSciences, Inc.
College Station, TX 77840 USA

Previously, this laboratory reported success in producing transgenic bovine fetuses (Biery et al., 1988, Theriogenology 29:224). Since that time, an effort was made to produce live, transgenic cattle. Data for two ova sources and four gene constructs are included. The first ova source was from excised oviducts of cattle stimulated with FSH. These ova were collected at 36 hrs post onset of estrus (24 hr post initial breeding). The second source of ova was IVM-IVF. IVM-IVF ova were subjected to pronuclear injection beginning at 18 hrs post IVF. Structural genes used were human estrogen receptor (HER) and insulin-like growth factor-I (IGF-I). Two promoters from the chicken $\alpha$-skeletal actin gene were utilized, consisting of 202 bp of the promoter (202-ASK) or the promoter and first intron of the actin gene (733-ASK). Approximately 2 Kb of the mouse mammary tumor virus $3'$ long terminal repeat promotor (MMTV) was also used. Injected ova were cultured in an oviducal cell co-culture. At the end of culture, morulae and blastocysts were transferred non-surgically to synchronous recipients. After calving, blood and tissue samples were analyzed for the presence of a transgene by Southern blot analysis.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Ova Source</th>
<th>No. Ova Collected</th>
<th>No. Ova Injected (%)</th>
<th>No. Ova Developing (%)</th>
<th>No. Preg./No. Abort</th>
<th>No. Live Calves</th>
<th>No. Transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASKHER</td>
<td>In-vivo</td>
<td>4150</td>
<td>1878 (45)</td>
<td>266 (14)</td>
<td>82/12</td>
<td>53</td>
<td>1</td>
</tr>
<tr>
<td>202-ASK-IGF-I</td>
<td>In-vivo</td>
<td>2668</td>
<td>1346 (50)</td>
<td>205 (15)</td>
<td>62/21</td>
<td>35</td>
<td>1</td>
</tr>
<tr>
<td>202-ASK-IGF-I</td>
<td>IVM-IVF</td>
<td>5142</td>
<td>2559 (50)</td>
<td>178$^b$ (7)</td>
<td>44/8</td>
<td>32</td>
<td>2$^b$</td>
</tr>
<tr>
<td>MMTV-IGF-I</td>
<td>In-vivo</td>
<td>538</td>
<td>246 (46)</td>
<td>38 (15)</td>
<td>14/5</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>MMTV-IGF-I</td>
<td>IVM-IVF</td>
<td>798</td>
<td>667 (84)</td>
<td>63 (9)</td>
<td>15/2</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>733-ASK-IGF-I</td>
<td>In-vivo</td>
<td>295</td>
<td>136 (46)</td>
<td>15 (11)</td>
<td>6/4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>733-ASK-IGF-I</td>
<td>IVM-IVF</td>
<td>5775</td>
<td>4374 (76)</td>
<td>293 (7)</td>
<td>92/34</td>
<td>51</td>
<td>1$^b$</td>
</tr>
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</table>

$^b$ Not transferred. These calves were stillborn or died within one day of birth.
Where are we now?

- Engineering cells and Cloning
- Transposons
- Zinc fingers
- Talens
- Lentiviral delivery
FugW vector with shRNA targeting Prp
Proof of Concept
Targeting myostatin by RNAi

Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. McPherron AC, Lawler AM, Lee SJ.
Double muscling in cattle due to mutations in the myostatin gene. McPherron AC, Lee SJ.
RNA interference provides a means of regulating gene expression by targeting genes for silencing in a sequence-specific manner through the design of short hairpin RNAs (shRNAs). The development of such techniques for modifying transgenic livestock that express shRNAs has tremendous potential for agriculture. Pictured here are cloned, transgenic bovine blastocysts established by Tesseme et al. (this issue) that express GFP in addition to an shRNA that targets the myostatin gene, a negative regulator of muscle growth.

Targeting Myostatin in Cattle

Production of Transgenic Calves Expressing an shRNA Targeting Myostatin

SUMMARY
Myostatin (MSTN) is a well-known negative regulator of muscle growth. Abnormalities in this gene inhibit muscle mass buildup. A gene encoding a transgene specifically targeted to skeletal muscle inhibition has been identified. This gene is targeted to skeletal muscle inhibition in a transgenic model using RNA interference to enhance myostatin knockdown efficiency. The target of this strategy is to produce animals with enhanced muscle mass.

INTRODUCTION
Myostatin (MSTN), an autocrine growth inhibitor of skeletal muscle, is a negative regulator of muscle growth. The goal of this study was to produce transgenic animals with increased muscle mass by inhibiting Myostatin expression using RNA interference. A transgenic mouse model was developed to test this strategy. The transgenic mouse was generated using a lentivirus vector containing a Myostatin-shRNA construct. The transgenic mouse model was used to test the efficacy of this strategy.

A) A transgenic calf is shown standing in a pasture.
B) A non-transgenic calf is shown standing in a pasture.
Last Few Years

- Long standing relationship with Texas A&M
- Collaboration was developed to continue to move the Tg Malaria program forward by re-establishing the founder line/herd of goats at Texas A&M
- Aim was to re-garner interest and ultimately support/funding to continue to move the program forward
Malaria is a Major World Health Problem

- Caused by protozoan parasites infection e.g. *Plasmodium*
  - *Plasmodium falciparum*: ~80% of cases/ ~90% of deaths
  - *Plasmodium vivax*
  - *Plasmodium ovale*
  - *Plasmodium malariae*

- Resides within liver and red blood cells – hidden from immune system

- However, circulating, infected red blood cells are broken down by spleen

- High rates of morbidity and mortality, especially among children and women in endemic areas
  - Incidence: 300-350 million / year
  - Death: 1.5-3.0 million / year

- Control of malaria becoming increasingly difficult:
  - Vector control: insecticide-resistance mosquito
  - Chemotherapeutic agents: drug-resistant parasite
  - Vaccine: candidate search (anti-sporozoite, anti-merozoite, transmission blocking)
A recombinant vaccine expressed in the milk of transgenic mice protects Aotus monkeys from a lethal challenge with Plasmodium falciparum


Malaria Vaccine Development Office, Laboratory of Protozoan Diseases, National Institute of Allergy and Infectious Diseases, Rocky Mountain Institute, MD 80920, and

Anasazi Vaccine Corporation, Flagstaff, AZ 86001

Contribution by Louis H. Miller, November 20, 2001

Two strains of transgenic mice have been generated that secrete into their milk a malaria vaccine candidate, the 42-kDa C-terminal portion of Plasmodium falciparum merozoite surface protein-1 (MSP142). One strain secretes an MSP142 with an amino acid sequence homologous to that of the FVO parasite line, the other an MSP142 whose internal 34-amino acid repeat region was replaced by a region homologous to the C-terminal repeat region of MSP142 from the FVO parasite line. Vaccination of a pool of homologous FVO parasite line and a group of control animals with MSP142-expressing milk from these animals were given at 5-week intervals over a period of 15 weeks. Vaccination of a pool of homologous FVO parasite line and a group of control animals with MSP142-expressing milk from these animals were given at 5-week intervals over a period of 15 weeks.

A vaccine to control malaria is a highly desirable public health tool to reduce morbidity and mortality in African children. It also appears technically achievable, with a number of promising candidates identified over the last few years, most of which have entered clinical trials. The vaccine candidate in this study, the 42-kDa C-terminal portion of Plasmodium falciparum merozoite surface protein-1 (MSP142), was designed to target the C-terminal repeat region of MSP142 from the FVO parasite line, which is homologous to the C-terminal repeat region of MSP142 from the FVO parasite line. Vaccination of a pool of homologous FVO parasite line and a group of control animals with MSP142-expressing milk from these animals were given at 5-week intervals over a period of 15 weeks.

Materials and Methods

A recombinant protein production. The generation of the FVO parasite line expressing transgenic 42-kDa C-terminal portion of Plasmodium falciparum merozoite surface protein-1 (MSP142) vaccine candidate candidate was accomplished by constructing an expression plasmid containing the MSP142 gene under the control of a strong promoter, and transfecting these cells with this plasmid. The transgenic plasmid was then inserted into the genome of the FVO parasite line, resulting in the transgenic FVO parasite line. Vaccination of a pool of homologous FVO parasite line and a group of control animals with MSP142-expressing milk from these animals were given at 5-week intervals over a period of 15 weeks.

Acknowledgments: The authors thank Augusto de la Fuente, Jr., for help in the preparation of the manuscript. This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and the National Cancer Institute, National Institutes of Health.
GM goats encoding malaria antigen
Retroviral mediated transgenic animals

- 62 transgenic animals representing cattle (8), goats (12), sheep (24) and pigs (18).

1 year of work with vast majority of the time involving gestation. Risk assessment studies showed no movement of transgene to surrogate and no unwanted viral recombination (RCL)
Current Status of GM Livestock

- The ability to efficiently produce genetically modified livestock for food and/or the production of human medicines and other useful products is here.

- The applications of this technology and potential benefit to animal agriculture, animal and human health are mind-boggling and limited only by our imagination.