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Feature Article: Applications of RNA interference-based gene silencing in animal agriculture

C. R. Long
K. J. Tessanne
M. C. Golding

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Technical Note: A simple and cost-effective means to regulate oxygen content of trigas incubators using liquid nitrogen

J. L. Moss
L. Borilla
P. J. Hansen

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From the President

Dear Colleagues,

Midyear greetings! Plans for the 37th Annual Conference of the IETS in Orlando, Florida, are on track. Pete Hansen and his organizing committee have been working hard on the local arrangements for the conference. Program co-chairs Eckhard Wolf and Ciro Barros have developed an exciting scientific program with the theme “Reproductive biotechnology at the interface between animal agriculture and biomedical research.” The conference will include two preconference symposia as well as a post conference workshop.

University of Florida colleagues, Cliff Lamb and Jose Santos, have organized an interesting preconference symposium, “Advances in bovine reproduction and embryo technology.” Fulvio Gandolfi and the Domestic Animals Biomedical Embryology (DABE) Committee will be holding the 2nd DABE Workshop, which will address “Plasticity, fate control, and therapeutic applications of domestic animal stem cells.” Naida Loskutoff and the committee on Companion Animals, Non-Domestic and Endangered Species (CANDES) are organizing a post conference workshop on “International Regulations and Requirements for the Import/Export of Reproductive Biomaterials: Embryos, Semen and Tissues.” Details about all of these programs, including the conference site and related Orlando activities will be posted on the IETS website. As always, your attendance and participation as an IETS member will make Orlando a successful and memorable conference. The deadline for submitting abstracts is August 2nd. Information for abstract submission is posted at http://www.iets.org/2011/.

In addition to plans for the 2011 annual conference, several initiatives have recently been completed or are well underway. In an ongoing effort to encourage interaction with affiliate societies, letters of invitation to the 2011 Annual Conference as well as society brochures have been distributed to several national and international organizations. Board member Andras Dinnyes has spearheaded this effort. PowerPoint slides have also been developed to advertise the Orlando conference. Please contact Debi Seymour at FASS if you plan to attend a scientific conference and you would be willing to distribute IETS brochures or display slides about the 2011 Orlando conference.

The new 4th edition of the IETS Manual, edited by David Stringfellow and Daniel Givens, is now available in CD format through the IETS website. This manual contains the latest information on the production, health, and disease transmission of embryos. More than 70 manuals have been sold to date. The Spanish and French versions of the manual should be available for sale later this year. Another project which is underway is the new IETS website thanks to the ongoing efforts of Matt Wheeler, Richard Fayer-Hosken, and the staff at FASS. The new website is currently being edited and populated with IETS information and should be launched in fall 2010.

In closing, remember to mark your calendars and biological clocks for a migration to Orlando, Florida, to attend the 2011 Annual Conference. I look forward to seeing you all in Orlando!

Best Regards,

Peter Farin
IETS President
To: IETS Members
From: Eckhard Wolf and Ciro M. Barros, 2011 program co-chairs
Subject: Call for Abstracts

On behalf of the IETS and the chairs of the Local Organizing Committee, Peter Hansen, we would like to extend a warm invitation to all IETS members to join us at the annual conference of the IETS, scheduled for January 8–12, 2011, in Orlando, Florida, USA.

The theme of the 2011 annual meeting program is

Reproductive Biotechnology at the Interface Between Animal Agriculture and Biomedical Research

The program topics include

• Follicular reserve,
• Growth factors and follicular development,
• Recent advances in in vivo and in vitro cryopreservation,
• Genetic engineering of livestock,
• From epigenetics to epigenomics, and
• Molecular networks as sensors and drivers of fertility.

There also will be a Practitioner’s Forum on Commercial IVF, and the program will conclude with a keynote address by Dr. Thomas Cremer titled “Higher order chromatin organization and nuclear architecture in developmental biology and cell specialization.”

The Local Organizing Committee is planning a variety of social events. Tours will be available before and after the meeting. There will be an opening reception on Sunday evening, and the closing banquet and activities will be on Tuesday evening.

Details about the program, including the invited speakers and titles of their presentations, are currently available on the IETS Web site:

http://www.iets.org/2011

In addition to the main program, on Saturday, January 8, 2011, there are plans to hold a preconference symposia on “Advances in bovine reproduction and embryo technology” organized by Dr. Jose Santos and Dr. Cliff Lamb and an afternoon preconference workshop titled “Current progress in domestic animals embryonic stem cell research” organized by Dr. Fulvio Gandolfi and the DABE committee. All of these events will be held at the Wyndham Orlando Resort in Orlando, Florida. Registration information will be posted on the Web site as soon as it is available.

We once again urge all members to use the electronic submission format to help keep costs to a minimum.

We sincerely hope that you will all be able to join us in Orlando to participate in IETS 2011!
IETS Annual Conference 2011
Wyndham Orlando Resort, Orlando, Florida
Co-Program Chairs: Eckhard Wolf and Ciro Barros
“Reproductive Biotechnology at the Interface between Animal Agriculture and Biomedical Research”

Tentative Program

Thursday, January 6, 2011

10:00–18:00 IETS Board of Governors Meeting

Friday, January 7, 2011

09:00–17:00 IETS Board of Governors Meeting
16:00–19:00 Registration

Saturday, January 8, 2011

07:00–18:00 Registration
11:00–18:00 Poster setup
08:00–17:00 Pre-conference symposium – Advances in bovine reproduction and embryo technology
13:00–17:00 IETS Foundation Board of Trustees Meeting
14:00–18:00 2nd DABE workshop: Plasticity, fate control, and therapeutic safety of stem cells
11:00–18:00 Commercial exhibit setup
17:00–18:00 IETS student group (The Morulas) meet and greet

Sunday, January 9, 2011

06:30–08:00 Poster setup
07:30–08:30 Past President’s breakfast
07:30–08:30 Student competition breakfast with Foundation Education Committee
07:00–018:00 Registration
08:00–017:00 Commercial exhibition
08:30–09:30 IETS Foundation Education Committee
08:45–09:00 Opening and welcome (E. Wolf and C. Barros)
09:00–10:30 Session I—Follicular reserve

09:00–09:45 Does size matter in females?
J. J. Ireland, Michigan State University

09:45–10:30 In vitro and in vivo control of follicle formation and activation in cattle
J. E. Fortune, Cornell University

10:30–11:00 Refreshment break/exhibition
11:00–12:30 IETS Foundation Student Competition Presentations
12:30–14:00 Lunch break
12:30–14:00 IETS Board luncheon with affiliate society representatives
12:30–14:00 Health and Safety Advisory Committee (HASAC)—Forms and Certificates Subcommittee

14:00–15:30 Session II: Growth factor and follicular development

14:00–14:45 Oocyte factors and follicle development
R. B. Gilchrist, University of Adelaide

14:45–15:30 Follicular somatic cells factors and follicle development
J. Burattini Jr., São Paulo State University

15:30–16:00 Refreshment break/exhibition

16:00–17:30 Session III: Recent advances in in vivo and in vitro cryopreservation

16:00–16:45 Cryopreservation and in vitro culture of caprine preantral follicles
J. R. de Figueiredo, LAMOFOPA
High-pressure treatment of oocytes and embryos to increase cryosurvival
_C. Pribenszky, Szent Istvan University_

Short presentations from submitted abstracts

Health and Safety Advisory Committee (HASAC) Open Meeting

Welcome reception

**Monday, January 10, 2011**

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
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<tbody>
<tr>
<td>07:30–16:00</td>
<td>Registration</td>
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<tr>
<td>08:00–18:00</td>
<td>Commercial exhibits</td>
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<tr>
<td>08:00–17:00</td>
<td>A/V library/speaker preparation</td>
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<tr>
<td>08:30–10:30</td>
<td>Poster session I/refreshment break/exhibition</td>
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<tr>
<td>10:30–11:15</td>
<td><strong>Session IV: Genetic engineering of livestock</strong></td>
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<tr>
<td>10:30–11:15</td>
<td>Transgenic livestock in agriculture and biomedicine—An update</td>
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<tr>
<td>11:15–12:30</td>
<td><strong>Session V: From epigenetics to epigenomics</strong></td>
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<tr>
<td>11:15–12:00</td>
<td>Epigenetic control of development and expression of quantitative traits</td>
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<tr>
<td>12:00–12:30</td>
<td>Short presentations from submitted abstracts</td>
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<tr>
<td>12:30–14:00</td>
<td>IETS Data Retrieval Committee Meeting</td>
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<tr>
<td>12:30–14:00</td>
<td>Lunch break</td>
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<tr>
<td>12:30–13:30</td>
<td>Exhibitors’ luncheon with the IETS Board</td>
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<tr>
<td>14:00–16:00</td>
<td><strong>Session VI: Molecular networks as sensors and drivers of fertility</strong></td>
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<tr>
<td>14:00–14:45</td>
<td>Holistic view of genomes and their transcriptome networks and aspects regarding fertility</td>
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<td>14:45–15:30</td>
<td>Dynamic proteome signatures in gametes, embryos, and their maternal environment</td>
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<tr>
<td>15:30–16:00</td>
<td>Short presentations from submitted abstracts</td>
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<tr>
<td>16:00–16:30</td>
<td>Refreshment break/exhibition</td>
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<tr>
<td>16:30–17:00</td>
<td>IETS Pioneer Award Presentation</td>
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<tr>
<td>17:00–18:00</td>
<td>IETS annual business meeting</td>
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<td>18:00–19:00</td>
<td>Companion Animal, Non-Domestic and Endangered Species (Candes) open meeting</td>
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<tr>
<td>18:00–19:00</td>
<td>Domestic Animal Biomedical Embryology Committee (Dabe) open meeting</td>
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**Tuesday, January 11, 2011**

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
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<tbody>
<tr>
<td>07:00–08:30</td>
<td>Organizational meeting of the IETS Board of Governors</td>
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<tr>
<td>06:00–07:45</td>
<td>9th IETS annual running competition</td>
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<tr>
<td>08:00–15:00</td>
<td>Registration</td>
</tr>
<tr>
<td>08:00–13:30</td>
<td>Commercial exhibits</td>
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<tr>
<td>08:00–17:00</td>
<td>A/V library/speaker preparation</td>
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<tr>
<td>08:30–10:30</td>
<td>Poster Session II/refreshment break/exhibition</td>
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<tr>
<td>10:30–12:30</td>
<td>Practitioners’ forum</td>
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<tr>
<td>12:30–13:30</td>
<td>Lunch break</td>
</tr>
<tr>
<td>12:00–13:30</td>
<td>Organizational lunch meeting of the IETS Foundation</td>
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<tr>
<td>13:30–17:00</td>
<td>Commercial exhibit and poster teardown</td>
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<tr>
<td>13:30–14:00</td>
<td>IETS Foundation Student Competition Awards, Candes and HASAC Updates, IETS Distinguished Service Award Presentation</td>
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<tr>
<td>14:00–14:45</td>
<td>IETS Distinguished Service Award Presentation</td>
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<tr>
<td>15:00–15:45</td>
<td><strong>Session VII: Keynote address</strong></td>
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<tr>
<td>15:00–15:45</td>
<td>Higher order organization and nuclear architecture in developmental biology and cell specialization</td>
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<tr>
<td>15:45–16:00</td>
<td>Closing ceremony</td>
</tr>
<tr>
<td>19:00</td>
<td>Closing party</td>
</tr>
</tbody>
</table>

**Wednesday, January 12, 2011**

<table>
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<tr>
<th>Time</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>08:30–17:00</td>
<td>Post conference, International regulations and requirements for the import/export of reproductive biomaterials: Embryos, semen, and tissues</td>
</tr>
</tbody>
</table>
Preparation of Abstracts for Poster Presentations  
2011 Annual Conference of the International Embryo Transfer Society (IETS)  
January 8–12, 2011  
Orlando, Florida

**General Information:** Free communication will be presented as posters, abstracts of which will be published in the January 2011 issue of *Reproduction, Fertility and Development*. The first author or a representative is required to present an expanded report of the data in poster format at the Annual Conference. Students may enter their abstract in a competition sponsored by the IETS Foundation and are eligible to compete for travel awards. Details on eligibility, judging procedures, and prizes are available on the IETS Web site.

**Abstract Requirements:** Abstracts must be in English and prepared strictly according to the instructions for submission. The language should be concise and should avoid jargon. Abbreviations must be defined and should be used sparingly. Abstracts should consist of unpublished, original data and contain objectives, experimental methods (including statistical methods), results, and conclusions. The program chairman and two other referees will subject each abstract to rigorous scientific review. Rejection by the program chairman is final. If withdrawal of an abstract becomes necessary, notify the program chairman by September 15, 2010.

**Submission Restrictions:** Due to space restrictions, submissions are limited. An individual may not be first author of more than one abstract. Space limitations allow a maximum of 3,250 keystrokes (including characters, spaces, and punctuation). Begin count at the title and end count with the last word. Abstracts that are too long will be rejected automatically by the system. Coding for special characters is not included in the total keystroke count. If you must use boldface, italic, or sub-/superscript type anywhere in the abstract, consult the Text Attributes Coding Information below. Special characters such as Greek letters and math symbols are available on the electronic submission form itself.

If your abstract includes a table, the keystroke limit will be 2,750 for the abstract and 500 for the table. The electronic submission form requires using the Table Wizard for preparation of tables. The printed abstract width allows for 70 keystrokes per line, including spaces; keep this in mind when formatting the table. Tables that are too wide are not printable. You must also include coding for text attributes in the table. This includes the italicization, superscripting, and subscripting of numbers and letters in the table and the footnotes.

**Submission Deadlines:** Abstracts must be submitted via the Web site only. No fax or e-mail copies will be accepted. The firm deadline for receipt of the abstract via the Web site is **August 2, 2010, by 11:59 PM Central Daylight Saving Time**. Abstracts received after this date will be rejected.

**Author Agreements:** By submitting an abstract, the author verifies (1) that the information in the submitted manuscript has never been published and is the work of the named authors, who all agree to be listed as coauthors, and (2) that the first author or a representative will present an expanded report of the data in poster form at the annual conference. Failure to present a poster after your abstract has been published in the conference proceedings can result in rejection of abstracts submitted by your laboratory in future years. Authors need to be aware of patent considerations and copyright considerations before submitting an abstract for publication.

All questions and correspondence should be directed to  
IETS Headquarters  
2441 Village Green Place  
Champaign, IL 61822 USA  
Phone: (217) 398-4697, Fax: (217) 398-4119  
E-mail: iets@assochq.org
Submission Procedures

Option I: Electronic Submission
Abstracts may be submitted electronically via the World Wide Web at

http://www.iets.org/2011

Instructions for submitting the abstract are available on the Web site. The firm deadline for receipt of the abstract via the Web site is August 2, 2010, by midnight CST. Abstracts received after this date will be rejected. After the deadline, it will not be possible to submit abstracts using the Web site. When you submit your abstract, you will be given a tracking number and a password. It is very important to keep a record of these, so please print the page with this information on it and keep it for your records. You may revise submitted abstracts anytime before the deadline, but you can only access your abstract using the password and tracking code. IETS headquarters will not have access to your tracking number and password if you lose them.

Be sure to mark your first and second section preferences on the submission form. Final selection of a section is left to the discretion of the program chairman. Section preferences:

- Student competition*
- Artificial insemination
- Cloning/Nuclear transfer
- Cryopreservation/Cryobiology
- Developmental biology
- Early pregnancy/Pregnancy recognition
- Embryo culture
- Embryo manipulation
- Embryo transfer
- Epidemiology/Diseases
- Exotic species
- Folliculogenesis/Oogenesis
- Gene expression
- IVF/IVP
- Male physiology
- Oocyte activation
- Oocyte maturation
- Sexing
- Sperm injection
- Stem cells
- Superovulation
- Transgenesis
- Ultrasonography

*This is for all those students in the Foundation Student Competition. A second preference MUST be chosen and the proper application forms submitted.

If you must use sub-/superscript type anywhere in the abstract, consult the Text Attributes Coding Information within this packet. Special characters, such as Greek letters and math symbols, are available on the electronic submission form itself in a character box to the right of the text area.

Important Note: If your abstract includes a table, please use the Table Wizard provided to submit it electronically. In addition to submitting the table electronically using the Table Wizard, you MUST fax, (217) 398-4119, a copy of the abstract to IETS headquarters so that we can verify the table layout. Please provide the author name, program section, and the tracking number on the printed copy of the abstract.
The use of figures and charts in abstracts is strongly discouraged. There is no electronic way to submit a figure. If your abstract must include a figure, in addition to submitting the abstract electronically, you must send one printed copy and a disk containing the figure to IETS headquarters to be received no later than July 29, 2010. The figure should be saved as an EPS file. Provide the name and version of the origination software, the operating system, the name of the first author, the file name(s), program section, and the abstract tracking number on the diskette label and the author name, program section, and the tracking number on the printed copy of the abstract.

Text Attributes Coding Information

When special characters or fonts, such as Greek characters, superscripts, or subscripts are needed, indicate these with the following TeX coding:

To make a character superscript, type \(^{3}\) (superscript 3 in this example)
To make a character subscript, type \(_{2}\) (subscript 2 in this example)
To make a character bold, format it like this: <b>word</b> the result will be word.
To italicize a character, format it like this: <i>word</i> the result will be word.
To underline a character, format it like this: <u>word</u> the result will be word.

Do not insert any extra spaces between the coding string and the adjacent characters in a word or expression. Do not use your word processor’s attribute functions to create bold, italics, or super-/subscripts. You must use the coding given above. Special math and Greek characters are available from the grid on the submission form itself; just click on the character you wish to insert. If your abstract includes a table, you must also include coding for text attributes in the table. This includes the superscripting and subscripting of numbers and letters in the table and the footnotes.

Please Note: Characters you choose from the boxes always appear at the end of the text already present in the abstract text area. If you have pasted your abstract into the text area from another word processor file, any special character you choose will appear at the end of your abstract. Simply highlight the code for this character then cut and paste it into the correct location in the abstract text.

The FASS Membership Department has recently made staffing changes to better serve the needs of our clients. Debi Seymour, FASS project manager, has taken over the executive secretary duties for IETS. Debi has been with FASS for over 12 years and has extensive experience in meeting planning and administrative duties to support IETS. Jennifer Gavel will remain on staff at FASS and will be available for historical information and references. FASS appreciates our partnership with IETS and looks forward to working with you in 2010 and beyond.
Introduction
Jose Santos, University of Florida; Cliff Lamb, University of Florida

Understanding the relationship between the follicle and embryo
Jo L. Leroy, University of Antwerp

Mechanism of uterine defense and control of uterine disease in cattle
Stephen Leblanc, University of Guelph

Controlling the dominant follicle
Milo Wiltbank, University of Wisconsin

Timed ET programs for management of donor and recipient cows
Pietro Baruselli, University of São Paulo

Influence of progesterone on oocyte quality and embryo development in cows
Pat Lonergan, University College Dublin

The role of cytokines and growth factors to improve pregnancy in IVF-ET programs
Jeremy Block, University of Florida

Dietary manipulations to improve embryonic survival in cattle
Willam Thatcher, University of Florida

What technologies are needed in the future of bovine reproduction and ET—Round table
Don Bennink, North Florida Holsteins; Brad Stroud, Embryo Service; Mel DeJarnette, Select Sires, Inc.

Conclusion
Jose Santos, University of Florida; Cliff Lamb, University of Florida
Plasticity, fate control and therapeutic applications of domestic animal stem cells

Orlando, Florida Wyndham Orlando Resort
January 8th, 2011

Welcome and opening remarks
Fulvio Gandolfi, University of Milano

Production of Oct4/GFP transgenic pigs: A new large animal model for reprogramming
Heiner Niemann, Institut Fuer Tierzucht

Mechanical phenotyping of embryonic stem cells
Carol Keefer, University of Maryland

Development of porcine neural progenitor stem cells for studying and treating Alzheimer's Disease
Vanessa Hall, University of Copenhagen and Poul Hyttel, Institut for Basal Husdyr

Equine amniotic derived stem cells: Progresses and perspectives
Fausto Cremonesi, University of Milano

The multi-potentiality of skin-derived stem cells in pigs
Randall S. Prather and Mingtao Zhao, University of Missouri

Markers of stemness in equine mesenchymal stem cells
Ann Van Soom, Ghent University

Strategies for regeneration of the bone using porcine adult adipose-derived mesenchymal stem cells
Matt Wheeler, University of Illinois

Porcine cardiac progenitor cells: A promising biomedical model
Fulvio Gandolfi, University of Milano
Scholarship opportunities are available for the third annual Laboratory Training Course in Oocyte and Preimplantation Embryo Molecular Biology, August 2010 in Philadelphia. This training course is dedicated to advancing the study of molecular mechanisms controlling mammalian oogenesis and preimplantation embryogenesis. A limited number of scholarships are available to cover course registration costs plus additional support for living expenses (up to $1,500 for allowable expenses). Applicants must hold or be pursuing MD or DVM degrees. The goal of the PREGER Scholarship Program is to advance research in mammalian oocyte and embryo biology by clinical scientists interested in human reproductive medicine, applied animal reproduction, or basic gamete and embryo biology. Please visit www.pregercourse.org to apply. The deadline to apply has been extended to June 30, 2010.
The goal of this workshop will be to present and discuss the current status of biosafety issues when transporting biomaterials from CANDES as well as humans and livestock for use in biomedical research and embryo production in vivo, in vitro and artificial insemination. Members of IETS and experts working at regulatory agencies and in relevant research will provide presentations that will cover a variety of topics that will include cloning and stem cell research. The workshop will cover issues such as the potential for disease transmission via reproductive biomaterials and methods to reduce those risks, the current status of international regulatory agencies in their biosafety guidelines for the import and export of these biomaterials, and suggestions for research or actions to overcome some of the hurdles that currently hampers use of reproductive biomaterials in biomedical and conservation science research. No proceedings articles will be provided; however, the IETS Committee on CANDES will prepare a publication summarizing the results of the workshop in a 2011 issue of the *Embryo Transfer Newsletter*. The proposed topics and suggested speakers for the program are as follows:

### Session I: International regulations for the transport of embryos, semen, and tissues from domestic and non-domestic livestock

<table>
<thead>
<tr>
<th>Time</th>
<th>Topic</th>
<th>Speakers</th>
</tr>
</thead>
<tbody>
<tr>
<td>08:00–08:45</td>
<td>USDA-APHIS guidelines and regulations regarding the transport of reproductive tissues versus live animals</td>
<td><em>Dr. Linda Penfold, White Oak Conservation Center and IETS CANDES Subcommittee Co-Chair; Dr. Bill White, USDA-APHIS</em></td>
</tr>
<tr>
<td>08:45–09:00</td>
<td>Discussion</td>
<td></td>
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<tr>
<td>09:00–09:45</td>
<td>OIE and international guidelines and regulations regarding the transport of reproductive tissues versus live animals</td>
<td><em>Dr. Larry Delver, IETS HASAC Regulatory Subcommittee Chair; Dr. Gabriela Mastromonaco, Toronto Zoo and IETS CANDES Technology Subcommittee Co-Chair</em></td>
</tr>
<tr>
<td>09:45–10:00</td>
<td>Discussion</td>
<td></td>
</tr>
<tr>
<td>10:00–10:30</td>
<td>Break</td>
<td></td>
</tr>
<tr>
<td>10:30–11:15</td>
<td>Biosafety issues regarding the transport of tissues for cloning and stem cell research</td>
<td><em>Dr. Andras Dinnyes, Szent Istwan University and IETS Board of Governors Member; Fulvio Gandolfi, Institute Anatomia of Domestic Animals, IETS DABE Committee Chair</em></td>
</tr>
<tr>
<td>11:15–11:30</td>
<td>Discussion</td>
<td></td>
</tr>
<tr>
<td>11:30–12:15</td>
<td>Risks and methods for reducing risks of transmitting infectious pathogens when transporting reproductive biomaterials</td>
<td><em>Dr. Ann van Soom, IETS HASAC Research Subcommittee Chair; Dr. Naida Loskutoff, Omaha’s Center for Conservation and Research, IETS CANDES Chair</em></td>
</tr>
<tr>
<td>12:15–12:30</td>
<td>Discussion</td>
<td></td>
</tr>
<tr>
<td>12:30–14:00</td>
<td>Lunch</td>
<td></td>
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</table>

### Session II: International guidelines and regulations for the import and export of reproductive tissues from rare or endangered livestock breeds and non-domestic species

<table>
<thead>
<tr>
<th>Time</th>
<th>Topic</th>
<th>Speakers</th>
</tr>
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<tbody>
<tr>
<td>14:00–14:45</td>
<td>Current trends and disease risk assessments for the development of biobanks for tissues from a diverse array of taxa, including rare domestic and non-domestic livestock</td>
<td><em>Dr. Bill Holt, Zoological Society of London, IETS CANDES Health and Safety Co-Chair; Dr. Matt Wheeler, University of Illinois, US National Animal Germ Plasm Technical Committee, and IETS Board Member; Dr. Laura Hungerford, FDA/University of Maryland School of Medicine</em></td>
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<tr>
<td>14:45–15:00</td>
<td>Discussion</td>
<td></td>
</tr>
<tr>
<td>15:00–15:45</td>
<td>CITES international guidelines and regulations regarding the transport of tissues from endangered species</td>
<td><em>Dr. Justine O’Brien, University of Sydney, Sea World, and IETS CANDES Regulatory Subcommittee Co-Chair; CITES Representative (to be named)</em></td>
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<tr>
<td>15:45–16:00</td>
<td>Discussion</td>
<td></td>
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<tr>
<td>16:00–16:30</td>
<td>Break</td>
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<tr>
<td>16:30–17:00</td>
<td>Panel discussion and final comments</td>
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The focus will be on suggestions on how to approach regulatory agencies for guidance to relax regulations that currently affect biomedical and conservation research programs—similar to the OIE’s acceptance of the embryo appendices in the Animal Health Code based on the efforts of the IETS HASAC.
Professor Whitten, the pioneer of in vitro embryo culture, passed away, but his legacy is very much alive in our laboratories

Written by Andras Dinnyes

Dr. Wesley Kingston Whitten, the recipient of the IETS Pioneer Award in 1996 and an outstanding scientist in reproductive physiology, passed away on May 24, 2010, in Canberra, Australia.

He was born in Macksville, New South Wales, and attended Sydney University, where he graduated in 1939 with a degree in veterinary science. Dr. Whitten served four years as a captain in the Australian Army Veterinary Corps and later joined Australia’s Commonwealth Scientific and Industrial Research Organisation to work on the reproduction of sheep. Between 1950 and 1961 he was a faculty member of the Australian National University, where his research focused on delayed implantation of lactating mice, making a seminal contribution to in vitro embryo culture. Furthermore, his findings pioneered the study of mammalian pheromones and their receptor, the vomeronasal organ. The influence of sexual pheromones on murine reproduction, his discovery, is known as the Whitten Effect. From 1966 to 1978, he was associate director at the Jackson Laboratory in Bar Harbor, Maine.

Dr. Whitten was a prolific scientific author of over 100 publications and associate editor of Biology of Reproduction and the Journal of Experimental Zoology. For his achievements, Dr. Whitten was made a Fellow of the Australian Academy of Science in 1982 and was given the Pioneer Award of the IETS in 1996. In 1993, Dr. Whitten was awarded the Marshall Medal from the Society for the Study of Fertility.

Dr. Whitten made a tremendous contribution to embryology in multiple species as he was the first to formulate a nutrient solution (Whitten’s Medium) that supported the in vitro development of mouse 2-cell embryos all the way to the hatching blastocyst stage. He reported his breakthrough discoveries in Nature in 1956. His discoveries triggered a new era of research. Previously, research into embryo development and metabolism was severely hampered because of the inability to grow embryos in vitro. Whitten’s Medium allowed the culturing of mammalian eggs for the study of oocyte maturation, fertilization, and embryo development. This research on embryo development had a major effect on research and applications in animals and humans, including many activities of IETS members. In vitro fertilization in animals and the human led to an increase in food production and more efficacious treatments for human infertility (ART), respectively.

Dr. Whitten was a great mentor and educator. His own words from 2001 still carry an important message for us about the nature of discoveries and true scientific spirit:

“...I expect that some of my audience hope to learn how to become successful researchers and all I can say with confidence is ‘be born lucky, work hard, and choose suitable projects and a conducive environment.’ The research should be within one’s own capabilities and within a time frame that allows adequate testing of hypotheses. Most of my contributions come from testing false hypotheses. In my early experiments, mouse embryos were slow to develop so I gave them extra oxygen and they all died; whereas, the controls survived. I reasoned that, if the controls were anaerobic, they would not be able to metabolize lactate. So, I tested embryos with this substrate only to find that they love the stuff! I was awed to be the first to witness the hatching of mammalian embryos in vitro. In 1955 I published a note in Nature describing the first chemically defined medium for embryos, which became known as Whitten’s Medium. In 1996, when I was given the Pioneer Award by the International Embryo Transfer Society, I was presented with a bronze sculpture done by a well-known Canadian artist that depicts this amazing event.”

We will miss the young spirit and wisdom of Professor Whitten.

The following link provided the biographical and historical details: http://www.mun.ca/2001report/index.php?includefile=menu/honour.php§ion=10&includefile1=content/honour/whitten.php
Applications of RNA interference-based gene silencing in animal agriculture

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Abstract. Classical genetic selection, recently aided by genomic selection tools, has been successful in achieving remarkable progress in livestock improvement. However, genetic selection has led to decreased genetic diversity and, in some cases, acquisition of undesirable traits. In order to meet the increased demands of our expanding population, new technologies and practices must be developed that contend with zoonotic and animal disease, environmental impacts of large farming operations and the increased food and fibre production needed to feed and clothe our society. Future increases in productivity may be dependent upon the acquisition of genetic traits not currently encoded by the genomes of animals used in standard agricultural practice, thus making classical genetic selection impossible. Genetic engineering of livestock is commonly used to produce pharmaceuticals or to impart enhanced production characteristics to animals, but has also demonstrated its usefulness in producing animals with disease resistance. However, significant challenges remain because it has been more difficult to produce animals in which specific genes have been removed. It is now possible to modify livestock genomes to block expression of endogenous and exogenous genes (such as those expressed following virus infection). In the present review, we discuss mechanisms of silencing gene expression via the biology of RNA interference (RNAi), the technology of activating the RNAi pathway and the application of this technology to enhance livestock production through increased production efficiency and prevention of disease. An increased demand for sustainable food production is at the forefront of scientific challenges and RNAi technology will undoubtedly play a key role.

Additional keywords: disease resistance, lentivirus, livestock, transgenic.

Introduction

Estimates of the world’s population indicate the total number of humans is approximately 6.77 billion, with an annual growth rate of 1.2% (http://www.census.gov/ipc/www/popclockworld.html, accessed 23 September 2009). Around the world, increasing human populations are placing additional demands on the limited productive land for the cultivation of crops and production of livestock. As the world population increases, the pool of agriculturally productive land decrease and the need to develop strategies to enhance the productivity of our livestock resources is constantly escalating. Global pressures to improve the production efficiencies of plant and animal agriculture are already beginning to rise and will very likely escalate to a crisis point in the not too distant future. Improvements in nutrition, management and classical genetic selection strategies have resulted in considerable progress in livestock production systems worldwide. However, the pace of human expansion is beginning to exceed the potential of these strategies, which are limited by the genetic capabilities of each species and the geographic/environmental conditions in which the animals are located. Thus, new ideas and novel agricultural practices must be adopted.

Given the genetic diversity that is available for selection and propagation, there are theoretical limitations to the production capacity of livestock (Long 2008; The Bovine HapMap Consortium 2009). In addition, it requires considerable time to identify animals with superior production traits, test the heritability of those traits and then disseminate those traits throughout the population. Although historically effective at improving production capacity of animals, genetic selection strategies have led to decreased genetic diversity and the propagation of undesirable recessive traits. This trend is clearly stated in a recently published quote from The Bovine HapMap Consortium (2009): ‘The recent decline in diversity is sufficiently rapid that loss of diversity should be of concern to animal breeders’. To counter this trend, international efforts in genetic conservation have been implemented (Mariante Ada and Egito 2002; Blackburn 2004). Moreover, it is impossible to use genetic selection mechanisms to acquire traits that do not exist in the population (i.e. the gaining of a novel genetic trait not previously associated with the species). Thus, in order to sustain a growing human population, it is essential to evaluate alternative approaches to improving livestock production through the application of interventions based on genetic engineering and other biotechnologies to improve...
production capacity and reduce the environmental impact of each animal.

One new development likely to play a major role in the future of agriculture is the silencing of the expression of specific genes through RNA interference (RNAi). Application of RNAi-mediated functional genomics is revolutionising the field in non-rodent species and allowing the study of gene function in livestock species to an extent not previously imagined. The application of RNAi technology appears nearly limitless and it can be applied to any number of agriculturally important areas. In the present review, we highlight the basics of RNAi biology and technology as they pertain to the future of animal agriculture, using examples relevant to livestock production systems.

**Biology of RNAi**

In the early 1990s, scientists attempting to alter flower colour in petunias discovered that endogenous genes could be silenced without alteration of the genomic sequence (Napoli et al. 1990). This began a cascade of events that would ultimately lead to the discovery of the causative agent of RNAi, namely double-stranded (ds) RNA (Fire et al. 1998). The discovery of the RNA-processing pathway now has the potential to greatly enhance experimental biology, improve agriculture and, most importantly, prevent and cure disease.

RNA interference is cellular process that likely originated as a eukaryotic defence mechanism against RNA viruses (Hannon 2002; Aravin et al. 2007a; Malone and Hannon 2009). To defend against parasitic nucleic acids from the outside world, as well as endogenous ones, eukaryotic genomes developed complex defence mechanisms to silence integrated and invading viruses. During the course of evolution, these defence mechanisms have been co-opted to regulate the expression of protein-coding genes and enhance genomic stability (Hannon 2002). Although several other reviews have focused on the ever increasing complexity of RNAi biochemistry (Carthew and Sontheimer 2009), here we will only provide a brief overview to clarify further discussions of the application of RNAi to efforts to enhance health and productivity of animal agriculture.

RNA interference is a classical biological response with a distinctive trigger: dsRNA. This dsRNA can come in the form of endogenous short interfering (esi) RNAs, regulatory non-coding RNAs processed to produce microRNAs (miRNAs) or experimentally delivered short interfering (si) RNAs and short hairpin (sh) RNAs (Paddison and Hannon 2003). Once introduced into the cell, each of these triggers (or their immediate precursors) are processed by the RNA III enzyme Dicer into siRNAs (Bernstein et al. 2001). These siRNAs contain unique termini, are typically 19–21 nucleotides in length (Tomari and Zamore 2005) and fit into the binding pocket of a clade of RNA binding proteins termed Argonaute proteins (Carmell et al. 2002; Song et al. 2004; Matranga et al. 2005). Argonaute proteins form the catalytic core of the RNAi-induced silencing complex (RISC; Liu et al. 2004, 2005a; Martinez and Tuschl 2004). Once bound by Argonaute, the process of RNA scanning begins searching for cellular RNAs that are homologous to the siRNA guide strand. Once a target is identified, the RNA is either cleaved (Liu et al. 2004), translationally repressed (Pillai et al. 2005) or serves as a platform to recruit epigenetic repressors to gene promoters (Ting et al. 2005), depending on the context. Regardless of the specific mechanism, the end result is silencing of gene expression.

Many discrete loci in the genome produce short dsRNAs that are recognised as a substrate by Dicer and processed into siRNAs (Watanabe et al. 2006, 2008; Tam et al. 2008). These natural dsRNAs are transcribed from either pseudogenes within the genome, bidirectional promoters or, most frequently, by endogenous retroviral elements (Aravin et al. 2007b). Transcription from these diverse loci produces esiRNAs that mediate regulation of protein-coding genes and viral transcripts via target cleavage. In the past decade, scientists have learned to deliver synthetic dsRNA precursor or siRNAs in both cell culture and whole-organism experimental systems (Tuschl et al. 1999; Elbashir et al. 2001a, 2001b). These chemically synthesised molecules mimic the endogenous ones, do not activate a cellular immune response the way long dsRNA does and can induce both transcriptional and post-transcriptional gene silencing, depending on the design of the RNA trigger (Paddison et al. 2002a). siRNAs homologous to the mRNAs of protein-coding genes or viral genomes have been used in a variety of experimental contexts to study diverse aspects of cellular biology and attenuate viral infection (McCaffrey et al. 2003; Paddison and Hannon 2003). Indeed, antiviral siRNA therapy may become one of the last lines of defence for terminal human immunodeficiency virus (HIV) patients (Coburn and Cullen 2002; Novina et al. 2002).

During the course of evolution, eukaryotic cells adapted components of the RNAi machinery to add an additional regulatory mechanism to the expression of protein-coding genes. This separate and distinct pathway is triggered by the production of non-coding RNAs located within introns of coding genes or as separate transcriptional units, which are processed into miRNAs (Okamura et al. 2007; Ruby et al. 2007). This pathway begins in the nucleus with transcription of the non-coding primary miRNA or pri-miRNA, which is carried out by polymerase (POL) II and results in the production of capped and polyadenylated transcripts (Kim 2005). The miRNAs that exist as independent transcriptional units are frequently located in clusters and are transcribed in accordance with the regulatory factors associated with their POL II promoters, just as protein-coding genes. Once the pri-miRNA unit is transcribed, it forms a complex dsRNA loop structure that is recognised and cleaved into a pre-miRNA by the DROSHA-DGCR8 or ‘microprocessor’ complex (Lee et al. 2003; Denli et al. 2004). This complex contains DROSHA, an RNase III enzyme that recognises the complex loop structure and cleaves it into a stem–loop structure. The miRNAs located within introns of coding genes enter the pathway at this step. These pre-miRNAs are transcribed as introns of a gene, then the pre-miRNAs are produced during normal intronic RNA excision and splicing (Okamura et al. 2007; Ruby et al. 2007). Once produced, this stem–loop is then exported from the nucleus by EXPORTIN and delivered to DICER for processing into a mature miRNA (Murchison and Hannon 2004; Kim 2005). Once produced, the guide strand from the miRNA is loaded into the RISC and homologous sequences are either cleaved or translationally suppressed (Bagga et al. 2005; Humphreys et al. 2005; Liu et al. 2005a, 2005b; Pillai et al. 2005).
Exogenous control of RNAi induction

As with chemically synthesised siRNAs mimicking esiRNAs, scientists have developed methods to mimic the stem–loop structure of pre-miRNAs. Early experiments used plasmids to deliver shRNA expression cassettes into cells that used simple POL III promoters to drive the production of transcripts containing an inverted repeat with a loop sequence in the middle. These constructs would transcribe a short RNA that would fold back to form a stem–loop or ‘hairpin’-like structure. This structure is recognised by DICER and cleaved to produce a mature siRNA (Paddison et al. 2002a, 2002b). Given that chemically synthesised siRNAs only offer transient gene suppression or ‘knockdown’, shRNAs became an attractive alternative to homologous gene targeting or ‘knockout’.

Traditional knockout technology via homologous recombination has been difficult in species other than mice and chickens because of a variety of factors. However, the major rate-limiting step is the lack of pluripotent stem cells. Although beyond the scope of the present review, technical difficulties and costs associated with the production of knockout livestock limit the usefulness of this technology in large animal species (Prelle et al. 2002; Niemann and Kues 2003; Wang and Zhou 2003). Fortunately, several studies have produced ‘RNAi mice’ that clearly demonstrate that the stably integrated shRNA expression vector can produce a knockdown phenotype that is functionally similar to the knockout (Carnell et al. 2003; Hemann et al. 2003). Surprisingly, one benefit of stably integrated RNAi over traditional gene targeting is that RNAi-inducing constructs that result in only partial gene suppression can produce phenotypes distinct from the knockout and thus provide more information on gene function. These factors, along with the enormous difference in costs associated with gene targeting vs. RNAi suppression, have made shRNA transgenics an attractive tool for molecular biologists studying gene function and developing transgenic animals and crops. The ability to generate RNAi transgenics is especially significant for model organisms like pigs, sheep and cattle, for which stem cells have yet to be derived and so for which gene knockout is only attainable using somatic cell nuclear transfer (SCNT) or cloning from a modified somatic cell line (Denning et al. 2001; Lai et al. 2002; Phelps et al. 2003; Kolber-Simonds et al. 2004; Richt et al. 2007).

With the development of shRNA plasmid-based technology, cultured mammalian cells suddenly became genetically tractable and thus open for use in genetic screens (Paddison and Hannon 2003). The incredible power of genetic screens fuelled the development of ever improving delivery systems and genome-wide shRNA libraries. Initial shRNA libraries were developed using POL III promoters to drive the expression of short stem–loop structures (Paddison et al. 2004). Although these constructs were certainly effective, they lacked the finesse and power of established POL II promoters, which can be tissue specific or inducible. To address these issues, shRNA constructs that mimic miRNAs were produced (Silva et al. 2005). Given that miRNAs could be produced by classic plasmid-based POL II promoters (Stegmeier et al. 2005), scientists now had the capacity to use inducible tissue-specific RNAi (Dickins et al. 2005, 2007). These next-generation miRNA-based shRNAs or shRNA mirs delivered more consistent and robust gene suppression than the simple hairpin loops and thus a library of shRNA mirs was built covering the compliment of protein-coding genes within the human and mouse genomes (Silva et al. 2005). These constructs are in wide use today and have been used effectively in individual gene knockdown studies, as well as in genome-wide screening strategies (Silva et al. 2008).

Regardless of consideration of the molecule triggering the RNAi response, one major issue that must be addressed when producing transgenic animals is the consequence of off-target effects (Rossi et al. 2008). Not until large-scale RNAi-based genetic screens were initiated did scientists become fully aware of the potential for exogenous siRNA or shRNA molecules to influence the expression of non-targeted genes (Ma et al. 2006; Guttman et al. 2009). Indeed, in several published RNAi screens, some of the strongest siRNAs identified have no significant effect on the gene they were designed to target (Ivanova et al. 2006). Yet, their ability to influence the biological process being studied is consistent. Three potential explanations for these off-target effects have been put forward and each has relevance to the production of transgenic animals.

First, overexpression of shRNAs in mammalian cells sometimes overloads the endogenous RNAi pathway, diminishing the ability of RISC to perform its ‘normal’ function (Grimm et al. 2005). Although the propensity for this to happen when using appropriate promoters is reduced, strong promoters producing shRNAs may stress the system and produce unforeseen phenotypes (Giering et al. 2008). Second, scientists still do not have a firm grasp of how the siRNA guide strand interacts with target mRNAs. Evidence to date suggests that pairing occurs strongest at the 5’ end of the siRNA, meaning that target specificity may be determined by the first 10 bp and not the full 21, implying that the specificity as determined by BLAST may not be as unique as desired (Schwarz et al. 2003; Matranga et al. 2005). Finally, most siRNAs and shRNAs to date have been designed to target the protein-coding component of the genome, virtually ignoring the non-coding fraction. A significant fraction of the genome is transcribed, yet only 1.5% is predicted to encode amino acid sequences (Lander et al. 2001; Carninci et al. 2005). Until recently, this non-coding component was thought to represent transcriptional noise; however, several recent papers have demonstrated conservation of the organisation and regulatory elements of these non-coding elements. Studies are now beginning to show that these non-coding RNAs regulate transcription of protein-coding RNAs and that siRNAs can inadvertently target these elements, as evidenced recently by identification of a non-coding RNA targeted by an ‘off-target effect’ siRNA (Guttman et al. 2009). A better understanding of the interaction between RISC and target mRNAs, as well as the processes that distinguish an RNAi molecule sorted into the siRNA pathway vs. the miRNA pathway, is needed (Rossi 2008). Until then, scientists must contend with off-target effects the traditional way: by running appropriate controls. Cell culture comparisons should be made to non-targeting controls such as scrambled siRNAs or shRNAs targeting genes not encoded by mammalian genomes. In the case of animal transgenics, careful consideration of the animal phenotype in relation to wild-type and non-targeting shRNAs must be made.

Continued on next page
Endogenous control of RNAi induction

Given the ubiquitous nature of RNAi, the study of functional genomics in agriculture has made several significant leaps forward. As we emerge from the genome-sequencing era and more gene sequence data become available, it is likely that we will see the development of shRNA libraries for large animal model organisms. Indeed, scientists have already started to use RNAi in making transgenic cells and animals to study gene function and to provide innate immunity to viral diseases (Chen et al. 2006; He et al. 2007; Lambeth et al. 2007; Wise et al. 2008; Joyappa et al. 2009; Li et al. 2009). Within this arena, the most significant challenge has been development of effective shRNA delivery systems, with the most popular of these being retroviral transgenesis (Pawlik et al. 1998; Singer and Verma 2008). Scientists have long used retroviral-based vectors to effectively deliver small transgenes into mammalian genomes. These vectors are perfectly suited to deliver shRNA expression cassettes into cultured cells and embryos. As with plasmid-based systems, the challenge remains finding adequate promoters to drive transcription in the cell type of interest. Viral promoters represent the most attractive choice because they work in a wide variety of mammalian cell types and species, as well as being usually quite robust in their output. The Human Cytomegalovirus Immediate-Early Promoter is perhaps the most commonly used. Another robust promoter in common use is the human or mouse elongation factor 1a promoter (EF1a) and, although very effective in embryonic tissues, it has been shown to decrease expression in differentiated adult tissues (Fleury et al. 2003). As with all transgenics, the choice of promoter must ultimately be made in accordance with the required level of transgene expression, the desired target tissue and developmental stage.

The biggest obstacle to retroviral transgenesis has been transgene extinction or silencing. This is a phenomenon that predominantly occurs when the integrated transgene is passed through the germline (Bestor 2000; Hofmann et al. 2006). Although founder animals may exhibit robust transgene expression, their F1 progeny will not, despite the fact that they possess an intact transgene. This transgene silencing is the result of an innate response to viral sequences discussed above. To contend with epigenetic gene silencing of integrated expression cassettes, scientists have started using recombinant lentivirus-based delivery systems (Lois et al. 2002). Unlike the first-generation gamma-retroviral systems, lentiviral expression vectors exhibit a lower incidence of silencing when passed through the germline and thus represent a means to produce transgenic livestock and F1 progeny that both contain and express the delivered transgene (Lois et al. 2002; Hofmann et al. 2003, 2004, 2006; Whitelaw et al. 2004; Pfiefer 2006).

The discovery of the RNAi biological pathway has tied together numerous aspects of transcriptional gene regulation, ranging from miRNA control of translation to RNA-directed gene silencing through chromatin modification. However, it is the use of RNAi as a genomics tool that will ultimately have the biggest impact on animal agriculture. Through several years of intensive research, scientists are now in possession of a significant number of molecular tools that enable the delivery of RNAi into embryos for the sole purpose of creating transgenic founder animals that carry resistance to disease or some other modified phenotype to enhance food or fibre production.

Improved production efficiency via RNAi

Increased muscle mass: controlling myostatin expression

Suppression of endogenous gene expression through RNAi in livestock species provides a valuable opportunity for improving economically important traits. A prime example of this is the myostatin gene. Myostatin, or growth differentiation factor 8, is a negative regulator of muscle growth. Cattle breeds such as Piedmontese and Belgian Blue have been shown to possess mutations that inactivate the myostatin gene (McPherron and Lee 1997; Berry et al. 2002). Therefore, these animals display a ‘double-muscled’ phenotype of dramatically increased muscle mass. This same result has also been demonstrated in other species, including sheep (Hofmann et al. 2006; Boman et al. 2009), dogs (Mosher et al. 2007), mice (McPherron et al. 1997) and even humans (Schuelke et al. 2004). The increase in muscle mass has been found to include both hypertrophy and hyperplasia of muscle fibres. Animals exhibiting this phenotype, although more muscular, are also prone to reduced female fertility and lower viability of offspring (McPherron and Lee 1997). However, the ability to manipulate the expression of this gene using RNAi would allow for modulation of gene function, thus controlling not only the degree of increased muscle mass, but also the time at which the suppression may occur so as to overcome the potential negative factors associated with the wild-type mutation.

Myostatin is a member of the transforming growth factor β superfamily of genes. It is first translated as a precursor protein consisting of a propeptide and a mature peptide joined by an RXRR cleavage site. Post-translational cleavage of this protein into the propeptide and mature forms, followed by dimerisation of the mature peptide and non-covalent binding to the propeptide, allows for regulation of action (Patel and Amthor 2005; Walsh and Celeste 2005). Dissociation from the propeptide is required for the mature form dimer to bind to its receptor, activin receptor type IIβ, and act through the Smad 2/3 pathway (Patel et al. 2005). Administration of a soluble form of this receptor or overexpression of the propeptide both mimic the effects of gene knockout, with increases in muscle mass (Patel et al. 2005; Walsh and Celeste 2005). In addition, in vitro experiments have demonstrated inhibitory effects of myostatin on muscle cell proliferation as well as differentiation. The addition of exogenous myostatin to myoblast cells in culture prevented progression from the G1 to S phase of the cell cycle (Thomas et al. 2000; Rios et al. 2001; Joulia et al. 2003). Furthermore, this treatment delayed differentiation of myoblasts into myotubes in both mouse and bovine cell culture, and this has been shown to be due, in part, to a downregulation of MyoD, a protein that plays a key role in determination of the myoblast lineage (Langley et al. 2002; Joulia et al. 2003). In 1997, McPherron et al. (1997) published the production of a mouse knockout model of myostatin. These mice display a distinct increase in muscle mass and differentiation, much like the double muscling seen in livestock species. Further studies in vivo using an shRNA targeting the mouse myostatin gene have
given similar results (Magee et al. 2006). Therefore, this technology should be adaptable to larger species. Indeed, work in our laboratory (K. Tessanne, unpubl. data) has shown the effectiveness of shRNAs targeting the myostatin gene. Different shRNAs designed against both the bovine and caprine myostatin genes were tested for their ability to decrease caprine myostatin mRNA levels in vitro. All four shRNA tests showed a marked decrease in myostatin mRNA levels, as analysed by quantitative real-time polymerase chain reaction (Fig. 1). This work is now being translated to expression in vivo through lentiviral transduction of bovine embryos to express these shRNAs. The expectation is that offspring produced this way will display increased muscle mass, much like animals with mutations in this gene. In addition, because these shRNAs do not completely eliminate expression of myostatin, the negative attributes of double muscling may be diminished depending on the degree of reduction of gene expression.

In addition to constitutive expression of these shRNAs for targeting endogenous gene expression, inducible systems may also be used in order to provide control over when they are expressed. Studies in mice have shown that the effects of targeting the myostatin gene can be induced postnatally. Repression of the myostatin gene in 4-month-old mice through a Cre/lox system resulted in a 26% increase in muscle mass after 3 months (Welle et al. 2009). Administration of an antibody against myostatin to adult mice also increased muscle mass (Bogdanovich et al. 2002). Work in Japanese Black cattle to determine myostatin levels following induced frostbite injury to muscle revealed a marked decrease in myostatin mRNA and protein levels, reinforcing this inverse relationship between increased muscle mass and myostatin expression after birth (Shibata et al. 2006). The use of an inducible system, such as a tetracycline-inducible promoter, to drive shRNA expression would provide a means of control over when gene repression occurs. Postnatal induction of shRNA expression would allow producers to stimulate increased muscle mass after birth and bypass fertility and viability issues commonly seen in double-muscled cattle.

**Improved disease resistance via RNAi**

*Endogenous gene silencing: prion protein*

RNA interference could also be used to manipulate the expression of other economically important genes. The reduction of expression of genes such as that for the prion protein (PrP) could provide an avenue for disease resistance in several livestock species. Prion-mediated diseases (transmissible spongiform encephalopathies) in livestock are potentially transmissible to humans, making them a concern in both livestock and human medicine. No cure is available for affected individuals; thus, disease prevention is critical to control. Previous murine studies have shown that reduction of PrP expression is sufficient to prevent infection following exposure to the pathogenic variant of PrP (Büeler et al. 1993; Sailer et al. 1994; Weissmann 1994). Subsequently, sheep and cattle have also been produced with targeted deletion of the prion gene (Denning et al. 2001; Richt et al. 2007). Although in vitro studies indicated that an RNAi-based approach would successfully decrease PrP, our laboratory demonstrated the proof-of-concept RNAi-based technique for silencing the expression of the goat PrP in vivo (Daude et al. 2003; Golding et al. 2006). Suppression of PrP by genetic engineering presents a reasonable approach for producing disease-resistant livestock without the risk of adverse phenotypes. Furthermore, suppression of prion production in livestock could inhibit transmission of prion diseases from animals to humans.

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**Fig. 1.** Suppression of recombinant caprine myostatin mRNA in vitro. Average fold change in caprine myostatin mRNA compared with GAPDH as an internal control after transfection with lentiviral plasmids containing short hairpin (sh) RNA cassettes. PG57, PG181, PG545, PG1026, plasmids containing shRNAs targeting myostatin; PGLUC, plasmid containing a nonsense shRNA targeting luciferase; no transfection: baseline expression of recombinant caprine myostatin in the HEK 293T cell line.
To decrease expression of PrP in vivo, we used SCNT using genetically modified caprine fibroblasts. An adult goat fibroblast cell line previously used in nuclear transfer (NT) experiments was infected with recombinant lentivirus carrying the construct encoding green fluorescent protein (GFP) and the PrP shRNA. Using the lentiviral approach resulted in high transduction efficiencies, with approximately 30% of the primary adult fibroblasts containing the stably integrated transgene, as evidenced by GFP expression. These cells were selected and used for SCNT to produce cloned transgenic goat embryos. Embryos were transferred into synchronised recipient females 24 h after NT. Overall, a total of 158 cloned embryos was transferred into eight recipients and resulted in one pregnancy (Golding et al. 2006). Thus, the pregnancy rates for this study are in line with those of previous studies of goat NT (Shen et al. 2006), as well as the results recorded previously in this cell line (M. C. Golding, unpubl. data).

To determine the capacity of the shRNA to silence gene expression in a disease-relevant manner in vivo, levels of PrP were evaluated in the brain. The caprine fetus was recovered surgically at 81 days gestation and tissues were harvested and compared with an age-matched control goat fetus. All tissues derived from the cloned fetus displayed strong GFP fluorescence consistent with the presence of the transgene. The ability of the shRNA to suppress PrP expression was analysed by western blot. Expression of PrP was reduced by over 90% in the transgenic fetus compared with that in the age-matched non-transgenic control (Golding et al. 2006). Thus, recombinant lentivirus-mediated transduction of adult-derived fibroblasts can generate stable cell lines from which to clone transgenic animals with markedly reduced expression of PrP, the causative agent of a neurodegenerative disease that can be transmitted to humans. More recently, a therapeutic strategy using lentiviral-mediated delivery of shRNA directly to the brain of scrapie-infected mice successfully reduced the level of PrP and extended the lifespan of treated mice (Pfeifer 2006; White et al. 2008). Thus, from both a prophylactic and curative perspective, the use of RNAi shows considerable promise for targeting endogenous disease-related genes in livestock.

Exogenous viral gene silencing: viral gene silencing
Foot-and-mouth disease virus

Foot-and-mouth disease (FMD) is a highly contagious disease of the Picornaviridae family of RNA viruses and one of the most dreaded livestock diseases, especially in countries that are currently FMD free. The economic implications for an outbreak of FMD are staggering and represent a significant concern for all citizens. In geographic areas where the FMD virus is endemic, mortality and morbidity of livestock and trade restrictions on the export of livestock products result in increased economic hardship for local producers and limit the access of the world’s population to food. The use of available vaccines is not common in non-endemic areas because the immune response to the vaccine interferes with the ability to distinguish vaccinated animals from those that may have become infected and capable of shedding the virus. This represents a real obstacle to establish FMD virus-free status in endemic areas or in countries recovering from outbreaks. Intense efforts have focused on developing novel vaccination strategies for this disease and next-generation vaccines may circumvent some of the existing limitations (Mason et al. 2003a; Grubman and Baxt 2004). However, the possibility exists to use genetic engineering to produce animals that do not require vaccination and exhibit an innate resistance to FMD virus. This approach could be highly advantageous in many areas worldwide where livestock are not readily accessible or local conditions restrict the proper storage, transport and injection of the vaccine. Furthermore, this approach would allow the use of current monitoring methodologies of the livestock population to identify the persistence of the FMD virus in the population without the complication of whether the animals have been vaccinated.

RNA interference-based strategies have benefited from the extensive sequencing and molecular characterisation of the FMD virus (Mason et al. 2003b, 2003c). In order to provide an effective block to a wide range of virus serotypes and strains, the genome sequences must be analysed using bioinformatics to identify genomic regions of high homology across strains. The sequence information can then be used to design siRNAs that will work efficiently across strains (Liu et al. 2005c). However, it takes as little as a single base substitution to negate the silencing capability of a single siRNA; thus, the development of strategies that target multiple homologous gene sequences will be necessary to obtain widespread therapeutic application of these technologies.

The application of RNA-induced silencing of FMD virus replication is being investigated intensely in several laboratories. To date, the ability of siRNAs to block FMD virus replication has shown promising results. In vitro studies using transient transfection of siRNAs specific to critical regions of the FMD virus genome have been shown to block FMD virus replication (Kahana et al. 2004; de los Santos et al. 2005; Li et al. 2009). In our laboratory, siRNAs to homologous regions of the FMD virus genome are also effective in blocking FMD virion replication in vitro, although the level of siRNA required for effectiveness is likely higher than that typically achieved from endogenous expression of shRNAs (M. Peoples, unpubl. data). This represents a concern for genetic engineering of livestock because the transgenic expression constructs used to express FMD virus-targeting shRNA may require high-level expression, which could lead to cytotoxic effects (Grimm et al. 2006). Careful consideration of the promoter and tissue specificity of the shRNA expression construct has been shown to alleviate these concerns (Giering et al. 2008).

In vivo studies targeting the FMD virus are ongoing and suggest that a resistant animal can be achieved. Early work focused on a therapeutic application of transient siRNA or plasmid-based expression of shRNA targeting the FMD virus. In both mice and guinea-pigs, siRNA or shRNA expression plasmids were capable of restricting FMD virus replication in vivo (Chen et al. 2004; Kim et al. 2008; Joyappa et al. 2009). The use of episomal vectors, such as adenoviral vectors, further expands the usefulness of shRNA in blocking FMD virus replication. These vectors can be administered and provide some protective effect within 24 h in guinea-pigs and swine (Chen et al. 2006). Although not completely effective to either homologous or heterologous strains of FMD virus, shRNA expression from the adenoviral vectors
sustains that transgenic technologies may be an effective method for enhancing livestock resistance to FMD virus. However, these vectors lack the ability to integrate into the animal’s genome and to be transmitted to the progeny to allow for long-term propagation of FMD virus-resistant livestock.

Recombinant retroviral vectors, especially lentivirus-based vectors, have been shown to be exceptionally effective as delivery mechanisms for shRNA-based gene therapy. As stated above, recombinant lentiviruses are capable of initiating integration of the transgene into the host cell genome and the transgene is protected from silencing by host cell genomic defence mechanisms. Thus, the use of lentivirus-based vectors provides a novel approach to the delivery of FMD virus-specific shRNA to produce animals with innate immunity to FMD. Our laboratory is currently developing lentiviral-based expression constructs that are capable of the high-level shRNA expression necessary for blocking FMD virus replication. Using this approach, transgenic embryos have been made and transferred either by direct injection of the recombinant lentivirus into the perivitelline space of the developing zygote or by transducing somatic cells and producing embryos by nuclear transfer (C. Long, unpubl. data).

**Equine infectious anaemia virus**

Equine infectious anaemia virus (EIAV) is a lentivirus and a classic example of the Retroviridae family, infecting equids with nearly a worldwide distribution. The EIAV is an RNA virus that is reverse transcribed to DNA inside the host cell and then integrates into the host DNA, causing a persistent lifelong infection. There is no effective vaccine, due primarily to the wide spectrum of antigenic variation in wild-type strains of the virus. There is no cure for the infected animal and, thus, the current method of control for an animal testing positive (Coggins test) is either lifelong quarantine or euthanasia (Leroux et al. 2004).

Using RNAi to block translation of the integrated provirus mRNA represents a significant challenge owing to the rapid evolution of the virus and the presence of distinct quasispecies within an individual animal. Therefore, our laboratory chose EIAV as a target for testing the capacity of RNAi to effectively block retroviral production *in vitro* (Craigio et al. 2006). To improve the effectiveness of the RNAi-induced silencing effect, we designed the shRNAs to interact with regions of the viral genome that are transcribed in polycistronic transcripts. Thus, degradation of the polycistronic message via RISC-mediated siRNA interaction would lead to decreased expression of multiple viral proteins simultaneously. To determine whether our transgenic approach could potentially alter EIAV replication in a previously infected animal, we used a persistently infected feline adenocarcinoma (FEA) cell line. These cells continuously produce EIAV and release it into the culture medium, allowing us to test supernatants of the cell-free culture medium for the presence and amount of EIAV via the reverse transcription (RT) assay.

Cells were exposed to a recombinant lentiviral vector containing an expression construct capable of producing red fluorescent protein (dsRed) and an shRNA targeting various regions of the EIAV genome. Transgenic cells were placed under G418 (neomycin analogue) selection until approximately 100% of cells expressed the dsRed marker and presumably the shRNA.

Four of these lines and the non-targeting control (targeting luciferase) were analysed for production of EIAV by the RT assay. Culture media were recovered and cell-free supernatants prepared for the RT assay. The RT assay results from preliminary experiments in the present study were normalised against the non-transgenic control and are shown in Fig. 2.

The results of the RT assay suggest that shRNAs targeting EIAV are initially very effective at suppressing viral replication 8 days following genetic modification. However, by Day 12 after transduction, EIAV levels were elevated in both Pol2 and Rev2 lines. Levels of EIAV in the culture medium continued to rise in these lines on Day 17 and significant increases in RT activity in both the SER2 and GP2 lines were observed in the present study. These data indicate that EIAV is capable of escaping the single shRNA-induced suppression, as expected. Whether the virus produced from these transgenic FEA cells is fully functional (i.e. infective and replication competent) remains to be determined. These data correspond well with other reports of targeting retroviruses, such as HIV in human cell lines (Hu et al. 2002; Lee et al. 2002; Boden et al. 2003; Das et al. 2004).

Owing to the continuous evolution of viral genomes to circumvent host defences, a combinatorial approach to RNAi-based silencing of viruses is warranted. Simultaneous targeting of multiple viral genes is one mechanism proposed, but may also require blocking of pathogen entry into cells by altering receptor expression, expressing decoy molecules to bind critical viral proteins or increasing the innate immune response to viral invasion (Ding et al. 2002; Akkina et al. 2003; Li et al. 2005a; Shi et al. 2007).

**Other virus targets of agricultural interest**

The promise of using RNAi as an antiviral defence has prompted an explosion of publications in an ever expanding variety of applications. From poultry to pigs, the application of RNAi-based therapeutics is beginning to show real promise in enhancing our ability to defend our agricultural animal resources against viral disease. The successful control of viruses in livestock populations also has profound effects on human medicine.

Recent work has demonstrated RNAi to be effective in eliciting stable suppression of the porcine endogenous retroviruses
The use of RNAi-based targeting of viral genes has proven to be effective against a host of different contagions in a wide range of species ranging from birds to humans. These successes demonstrate that the strategy of targeting viral genes can effectively give the host an advantage in combating viral infections. It is important to consider that the innate and humoral immune response of the animal will also contribute to the suppression of virus replication. Together, enhancement of the animal genome and priming of the immune system through vaccination can potentially overcome many diseases, leading to a healthier, more prolific livestock production system. Furthermore, augmenting the disease resistance capacity of livestock will not only remove a significant reservoir of viral agents potentially affecting human and wildlife populations, but it will also increase the number of animals eligible to be sent to market. Continued development of more tunable tissue-specific transgene expression systems will likely enable the production of refined and better-controlled animal transgensics that will push agricultural production to the levels needed to feed the 22nd century.

As we move into the future, it is essential that the debate over the development and use of transgenic animals be initiated and conducted with careful consideration of the facts surrounding animal transgenics and their potential impact on human health and disease. Although both sides of the biotechnology debate have valid concerns, we simply cannot ignore the pressures that the increased population of tomorrow will place on the food and water supply. Adoption of biotechnology into agricultural practice is one method to alleviate these pressures and help maintain the environment. The production of disease-resistant animals with low phosphorous waste and increased milk, meat and fibre production are attainable goals through genetic engineering that will increase production levels to satisfy the demands of tomorrow’s populations. Within this realm, the field of RNAi is likely to play a major role.

Summary

The use of RNAi-based targeting of viral genes has proven to be effective against a host of different contagions in a wide range of species ranging from birds to humans. These successes demonstrate that the strategy of targeting viral genes can effectively give the host an advantage in combating viral infections. It is important to consider that the innate and humoral immune response of the animal will also contribute to the suppression of virus replication. Together, enhancement of the animal genome and priming of the immune system through vaccination can potentially overcome many diseases, leading to a healthier, more prolific livestock production system. Furthermore, augmenting the disease resistance capacity of livestock will not only remove a significant reservoir of viral agents potentially affecting human and wildlife populations, but it will also increase the number of animals eligible to be sent to market. Continued development of more tunable tissue-specific transgene expression systems will likely enable the production of refined and better-controlled animal transgensics that will push agricultural production to the levels needed to feed the 22nd century.

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References


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http://www.publish.csiro.au/journals/rfd
A simple and cost-effective means to regulate oxygen content of tri-gas incubators using liquid nitrogen

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Preimplantation embryos are often cultured in a low (5%) oxygen atmosphere. An atmosphere of 5% carbon dioxide (CO₂), 5% oxygen (O₂), and 90% nitrogen (N₂) (vol/vol) is typically achieved in a tri-gas incubator by injecting CO₂ and N₂. Given the large volume of gas required, nitrogen is consumed more rapidly than CO₂. In a very active laboratory, nitrogen cylinders must be replaced frequently. In our laboratory, for example, about 4 cylinders of compressed N₂ are used each week, whereas CO₂ cylinders are replaced at about monthly intervals. Frequent replacement of cylinders is time consuming and expensive. Moreover, the risk that embryo cultures will be compromised because of depletion of a gas cylinder increases as the rate of replacement of cylinders increases. Here we describe the use of liquid N₂ (LN₂) vessels to simplify the management of N₂ supply in an embryo culture laboratory.

In this system, 180-L storage vessels of LN₂ that contain a pressurized vapor bleed-off outlet are used to deliver gaseous N₂ to the incubator. The particular vessel used in our laboratory is a Dura-Cyl MCR 180 MP from MVE (Chart Industries, Ball Ground, GA, USA), which we rent from a local LN₂ provider. This tank delivers N₂ gas at up to 230 PSI to one of its outlets labeled “Gas Use” through a gas withdrawal valve (V in Figure 1). A standard pressure regulator that is used on nitrogen gas cylinders (R in Figure 1) is attached to the outlet fitting (F in Figure 1). This outlet fitting (F) is a standard Compressed Gas Association (CGA) connection (CGA 580) for nitrogen pressure regulators. The output from the pressure regulator is set at 10 PSI and sent directly to the incubator’s “A” input to be the primary nitrogen source. The incubator switches to a “B” input line connected to a standard compressed nitrogen gas cylinder when the input pressure from “A” gets low.

In our hands, a single 180-L LN₂ storage vessel lasts about 30 days. We have seen no reduction in embryonic development after replacing compressed N₂ cylinders with LN₂, and there has been a substantial reduction in direct costs for nitrogen. In a month, we typically used about 28 cylinders of industrial-grade N₂ at a cost of $72.00. If we had used high-purity nitrogen, the cost would have been $432/month. We obtain 180 L of LN₂ (a month’s supply) for $31.00. In addition, less labor is occupied with switchovers (one versus 16 per month), and there are fewer opportunities for the nitrogen supply to be depleted while embryos are being cultured.

One caveat is that the liquid vessels stay cold by evaporative cooling and venting off of nitrogen gas. Thus, the LN₂ will be consumed even if N₂ is not being delivered to incubators. Accordingly, LN₂ tanks should not be used as a backup supply of N₂ and are not advised when the monthly use of N₂ gas is low.
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June 2010
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14th Annual Conference of ESDAR and the 22nd Annual meeting of EU-AI-Vets

Dear Colleagues,

On behalf of the Local Organising Committee, I would like to extend a warm invitation to join us at the 14th Annual Conference of ESDAR and the 22nd Annual meeting of EU-AI-Vets, to be held in Eger/Hungary between 15 and 18 September 2010.

The scientific program is available at: www.esdar2010.org.

An interesting and colorful range of social events will enhance the opportunities for delegates to network with associates as well as to enjoy the Hungarian cultural heritage. Fascinating pre and post conference tours will also be arranged to highlight the best what Hungary has to offer.

Looking forward to seeing you in Eger this September.

Yours sincerely,

Prof. Laszlo SOLTI
president ESDAR
president of Local Organising Committee
rector of Szent István University