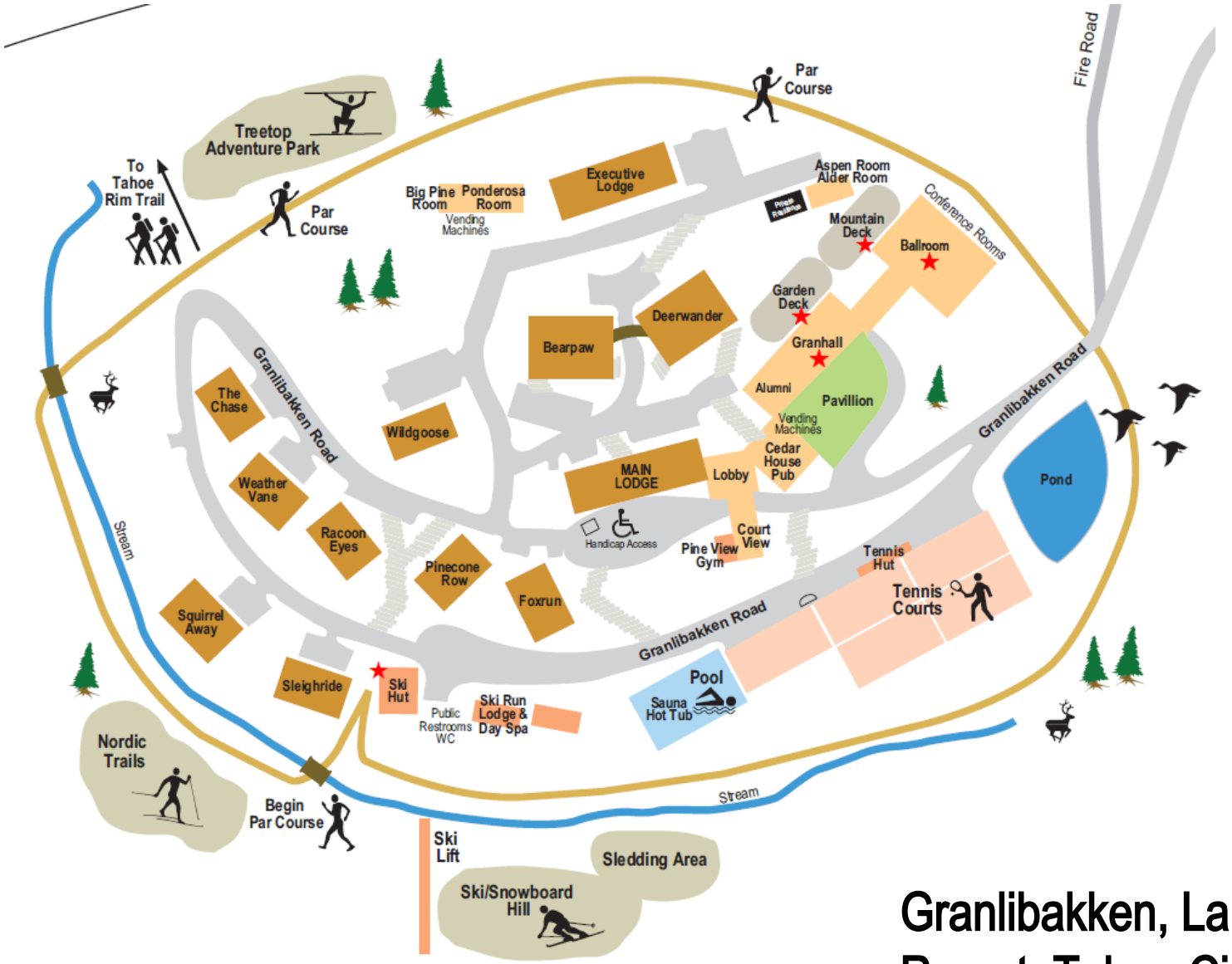
Conference*Program*

TRANSGENIC ANIMAL RESEARCH CONFERENCE XIV

August 13–17, 2023

Granlibakken Conference Center

Tahoe City, California



Granlibakken, Lake Tahoe Resort, Tahoe City

Transgenic Animal Research Conference XIV

Sunday-Thursday, August 13-17, 2023

Granlibakken, Lake Tahoe Resort, Tahoe City

Book of Abstracts

https://na.eventscloud.com/2023tarcucdavis/

UNIVERSITY OF CALIFORNIA
Agriculture and Natural Resources

UCDAVIS
DEPARTMENT OF ANIMAL SCIENCE

Transgenic Animal Research Conference XIV

Sunday-Thursday, August 13-17, 2023, Granlibakken, Lake Tahoe Resort

WELCOME

Welcome to the fourteenth in a series of conferences focusing on the genetic engineering of non-murine animals and their potential applications in agriculture and medicine. We are excited by the breadth and scope of the topics being addressed and the many recent developments around gene editing technologies should make the plenary and poster sessions interesting and informative. Work by graduate students and postdocs will be highlighted on the first day of the conference where during the second plenary session, each will participate in a poster pitch by presenting a summary of the poster they will be presenting. In addition, the diversity of backgrounds and interests of the participants and the format of the conference should provide many opportunities for stimulating interactions. The free afternoons on Monday and Wednesday will allow participants to take advantage of the recreational activities and scenic beauty available in the Lake Tahoe region, while talking with colleagues from around the world. We hope that you will find this conference enlightening and enjoyable and we look forward to your active participation.

UNIVERSITY OF CALIFORNIA
Agriculture and Natural Resources

UCDAVIS
DEPARTMENT OF ANIMAL SCIENCE

General Information

LOGISTICS:

Please check the inside cover of this program to see the location of rooms and facilities. All plenary sessions will be held in the Mountain/Lake Conference room. Poster and exhibits will be in the Pavilion. Please put posters up on the assigned board on Sunday night or Monday morning and take them down Wednesday evening.

Breakfast will be served from 7:00 to 8:00 am Monday through Thursday in Granhall. Coffee breaks will be on the Mountain Deck. Lunch and dinner will be served either on the Garden Deck or in Granhall. Messages may be left and retrieved on the message board outside the conference room. **Please note that your registration does not cover incidental charges such as telephone use within your rooms. There is a surcharge on all non-local calls.**

RECREATION:

There are many excellent recreational amenities at Granlibakken and within the surrounding region. At Granlibakken, facilities include a swimming pool, tennis courts and the Tree Top Adventure Walk with zip-lines. Offsite, on Monday and Wednesday afternoons you can enjoy river rafting, golf, Palisades Tahoe Aerial Tram ride, parasailing, stand-up paddle, or hiking on the many trails in the area. **Please note**, these excursions and activities are not part of the registration package and additional charges apply. We will endeavor to provide information and sign-up sheets for these activities outside the conference room. In addition, we will try to provide transportation to and from these activities for groups. **Please know that our student drivers need to be back onsite by 4 pm to participate in the poster sessions**.

Network: Granlibakken Wireless
No password required

UNIVERSITY OF CALIFORNIA
Agriculture and Natural Resources

UCDAVIS
DEPARTMENT OF ANIMAL SCIENCE

Table of Contents

UNIVERSITY OF CALIFORNIA
Agriculture and Natural Resources

UCDAVIS
DEPARTMENT OF ANIMAL SCIENCE

Thank You to Our Sponsors

Department of Animal Science, University of California, Davis

Agriculture and Natural Resources, University of California

W.K. Kellogg Endowment

The Roslin Institute, University of Edinburgh, UK sponsor of the John Clark Student Fellowship

Grant Support

This conference is supported by the Biotechnology Risk Assessment Grants (BRAG) Program Conference grant #2023-33522-40412-0 from the USDA National Institute of Food and Agriculture.

USDA National Institute of Food and Agriculture
U.S. DEPARTMENT OF AGRICULTURE

Planning Team

Program Committee UC Davis

Elizabeth Maga, Chair, Department of Animal Science
Anna Denicol, Department of Animal Science
Jim Murray, Department of Animal Science
Alison Van Eenennaam, Department of Animal Science

Planning and Logistics UC ANR, Program Support Unit:

Sherry Cooper
Mariette Malessy
Julia Kalika

UNIVERSITY OF CALIFORNIA
Agriculture and Natural Resources

UCDAVIS
DEPARTMENT OF ANIMAL SCIENCE

Agenda

Sunday, August 13

2-5 PM **Registration**, *Granlibakken, Lake Tahoe Resort*

5:30 **Hosted Hospitality Hour** (hosted bar)**,** *Garden Deck*

6:30 PM **Get-Acquainted Dinner** (no-host cash bar), *Garden Deck*

Monday, August 14

All PLENARY SESSIONS IN MOUNTAIN/LAKE ROOM

7-8 AM **Breakfast Buffet,** *Garden Deck*

8:00 **Welcome**
Elizabeth Maga, University of California, Davis, USA

SESSION 1 Technology
Session Chair: Pablo Ross, STgenetics, USA

8:10 Pablo Ross, STgenetics, USA
Opening Talk: Setting the Stage

8:40 Maciej Maselko, Macquarie University, Australia
Transgenic Animals Equipped with Microbial Enzymes for Bioremediation

9:20 Carlos Pinzón-Arteaga, University of Texas Southwestern Medical Center, USA
Blastocyst and Embryo like Structures Derived from Pluripotent Stem Cells Cultures

10:00 **Break***, Mountain Deck*

SESSION 2 Birds
Session Chair: Tae Hyun Kim, Penn State University, USA

10:30 Jae Yong Han, Seoul National University, South Korea
Genome Edited Chickens: Model for Production of Recombinant Proteins

11:00 Hicham Sid, Technical University of Munich, Germany
Transgenic Chickens Re-expressing Evolutionarily Lost Immune Sensors: Spotlight on the Evolution of the Avian Immune System

11:30 Poster Pitches

12 PM **Lunch,** *Garden Deck*
Free Time until 4:00 PM

4:00-5:30 **Poster Session I** (hosted beer and wine)**,** *Pavilion*

6:00 **Dinner** (no-host cash bar)**,** *Garden Deck*

SESSION 3 Agricultural Applications

Session Chair: Goetz Laible, AgResearch, New Zealand

8:00 Kiho Lee, University of Missouri, USA
Production of Genome Edited Pigs is Supported by Recent Technological Advancements

8:40 Dan Carlson, Recombinetics/Acceligen, USA
Thamani Dairy Cattle

9:20 PM Michela Ciccarelli, Washington State University, USA
Genetic Engineering of Reproductive Performance in Livestock

Tuesday, August 15

7:00-8:00 AM **Breakfast Buffet,** *Garden Deck*

ALL PLENARY SESSIONS IN MOUNTAIN/LAKE ROOM

SESSION 4 Products to Market, Session

Chair: Mark Walton, AquaBounty Technologies, USA

8:00 Mark Cigan, Genus, USA
Large Scale Generation of Commercially Elite PRRS Virus Resistant Pigs using CRISPR-Cas

8:40 Caitlin Cooper, Hendrix Genetics, USA
Methodologies for Setting Up and Conducting On Farm Trials for Practical Data

9:20 Keitaro Kato, Kindai University, Japan
Application of Genome Editing Technology to Marine Fish Aquaculture

10:00 **Break***, Mountain Deck*

SESSION 5 Regulation-Policy

Session Chair: Jim Murray, University of California, Davis, USA

10:30 Diane Wray-Cahen, USDA, USA
Enabling Regulatory Policies for Animal Biotechnology: A Global Overview

11:10 Daniel Kovich, USDA, USA
Animal Biotechnology and International Trade: Key Considerations

12:00-1:30 PM **Lunch,** *Garden Deck*

SESSION 6 Regulation-Journeys

Session Chair: Matthew Wheeler, University of Illinois, Urbana-Champaign, USA

1:30 Eric Schulze, UPSIDE Foods, USA
Regulating Biotechnology Used for Food: A Perspective on Policy from Inside FDA and from the Startup Community

2:10	John Buchanan, Center for Aquaculture Technologies, USA *Sterility is a Foundational Trait for the Large-Scale Commercialization of Genome Editing and Genetic Biotechnologies for Aquatic Species*
3:00	**Break**, *Mountain Deck*

SESSION 7 Regulation Journeys

Session Chair: Simon Lillico, Roslin Institute, UK

3:30	Chantal March, AquaBounty, Canada *Working in the Regulated Environment in the US, Canada and Internationally: AquaBounty's Experience*
4:00	Clint Nesbitt, Genus, USA *PRRSV-resistant Pigs: Genus PLC Global Regulatory Strategy and Experience*
4:30	John Bianchi, Revivicor, USA *GalSafe™ Pig Regulatory Journey*
5:30-6:30	**Hosted Hospitality Hour** (hosted bar)**,** *Garden Deck*
6:30-8:00 PM	**Dinner** (no-host cash bar), *Garden Deck*

Wednesday, August 16

7:00-8:30 AM	**Breakfast Buffet**, *Garden Deck*

ALL PLENARY SESSIONS IN MOUNTAIN/LAKE ROOM

SESSION 8 Agricultural Applications II

Session Chair: Anna Denicol, University of California, Davis, USA

8:00 AM	Aspen Workman, USDA, USA *First Gene-Edited Calf with Reduced Susceptibility to a Major Viral Pathogen*
8:40	Goo Jang, Seoul National University, South Korea *Production and Germline transmission of MSTN Gene Edited Cattle*
9:20	Diego Robledo, Roslin Institute, United Kingdom *Improving Disease Resistance in Aquaculture: Integration of Genome Editing into Breeding Programs*
10:00	**Break,** Mountain Deck

SESSION 9 Biomedical Applications

Session Chair: Kristin Whitworth, University of Missouri, USA

10:30	Tatiana Flisikowska, Technical University of Munich, Germany *Humanized Minipigs to Assess Drug Safety*
11:00	Mary Garry, University of Minnesota, USA *Humanized Skeletal Muscle: Novel Models and Therapies*
11:30	Irina Polejaeva, Utah State University, USA *Development and Characterization of a Novel Sickle Cell Disease Sheep Model*
12:00	**Lunch,** *Garden Deck* **Free Time until 4:00 PM**
4:00	**Poster Session II** (with hosted beer and wine)**,** *Pavilion*
5:30	**Dinner** (no-host, cash bar), *Garden Deck*

SESSION 10 Closing Session
Session Chair: Kevin Wells, University of Missouri, USA

8:00	Eric Hallerman, Virginia Tech University, USA Closing Talk: *Summary and Future*
9:30	General Discussionp
10:00 PM	Adjourn

Thursday, August 17

7-9 AM	**Breakfast Buffet**, *Garden Deck*
	Depart

Speaker Abstracts

Session 1: Technology

Chair: Pablo Ross, STgenetics

Setting the Stage

Pablo Ross, STgenetics, USA

Abstract not submitted.

Transgenic Animals Equipped With Microbial Enzymes for Bioremediation

Maciej Maselko, Macquarie University, Sydney, Australia

Pollution from industrial, urban, and military activity results in contaminated land and waterways that are not suitable for farming, drinking/irrigation, and recreation activities. Some compounds are biomagnified moving up trophic levels and can result in animals that are hazardous to consume. This is exemplified by fish with high concentrations of methylmercury and PFAS. Humans must limit their intake of fish harvested from contaminated areas and wildlife populations are negatively impacted.

Bioremediation methods using natural or genetically engineered microbes, plants, or enzymes in contaminated environments has been successful in some circumstance. However, microbes are not seldom very metabolically active in situ, free enzymes are unstable and/or require co-factors, transgenic plants and microbes are hard to bio-contain, and pollutants are often trapped within an organic matrix and not accessible. These limitations could be overcome by the deployment of animals engineered for bioremediation. Animals are highly metabolically active in situ, are readily bio-contained using physical, surgical, or genetic methods, and have digestive processes capable of extracting pollutants from an organic matrix. I will present data on exploring the potential of using engineered animals for bioremediation. Using fruit flies (Drosophila melanogaster), we test the scope of fungal and microbial enzymes that can expand animal metabolism. A wide variety of enzymes were functionally expressed, including those enabling flies to degrade/detoxify hazardous compounds. Fungal laccases and microbial mercury detoxification enzymes were expressed in transgenic zebrafish (Danio rerio) and their function evaluated in vitro and in vivo. Our work demonstrates that engineering animals to express fungal and microbial enzymes enables them to efficiently degrade/detoxify hazardous compounds and withstand higher concentrations than their wild-type counterparts. Deploying engineered animals may provide ecologically friendly and economical approaches for bioremediation of contaminated areas and disrupting the biomagnification of hazardous chemicals.

Blastocyst and Embryo Like Structures Derived from Pluripotent Stem Cell Cultures

Carlos A. Pinzón-Arteaga, Lizhong Liu, Jun Wu, University of Texas Southwestern Medical Center, Dallas

Understanding the molecular and epigenetic mechanisms behind blastocyst formation and implantation is of critical importance for improving the efficiency of assisted reproductive technologies. Advancements in the understanding of the signaling mechanisms that govern pluripotent stem cells

(PSCs) self-renewal and differentiation, together with small molecule compounds that modulate these pathways, have allowed to understand a range of pluripotency states, from 'Naïve' ESCs that resemble the pre-implantation epiblast to 'Primed' ESCs that correspond to the post-implantation epiblast. We have developed strategies for the generation of blastocyst like structures (Blastoids) from PSCs in a 3D culture environment. These blastoids, derived from 'Naïve' or 'Naïve-like' ESC cultures, or through self-organization of trophectoderm (TSCs) and PSCs, closely resemble blastocysts in terms of morphology, size, cell number, lineage composition and marker allocation. Blastoids offer a platform to apply high-throughput research techniques, thereby enhancing our understanding of the sequence in which various transcription factors appear, and the genetic, metabolic, and epigenetic processes that dictate early embryonic development. Lastly, the culture conditions we derived for blastoid growth have facilitated human PSCs to self-organize into embryo-like structures, termed 'peri-gastruloids'. These structures recapitulate critical stages of early human development, such as the formation of amniotic and yolk sac cavities, development of embryonic layers, specifying primordial germ cells, and initiation of early organogenesis.

Session 2: Birds

Chair: Tae Hyun Kim, Pennsylvania State University, USA

Genome Edited Chickens: Model for Production of Recombinant Proteins

Jae Yong Han, Kyung Min Jung, Jin Se Park. Major of Biomodulation, Animal Science and Technology, Department of Agricultural Biotechnology, Seoul National University, Korea

Chickens have been considered as efficient platform for production of recombinant proteins because of its high egg laying rate and ability to accumulate abundant proteins in the eggs. Also, the N-glycosylation pattern of egg proteins is beneficial to efficacy of certain biopharmaceuticals and human-like N-glycosylation pattern of egg proteins makes chickens as one of the optimal platform for production of recombinant therapeutic proteins. Recent advancements in CRISPR/Cas9 technology, combined with cultured PGCs, have revolutionized chicken genome editing, enabling the creation of valuable avian models and an egg bioreactor system. Using CRISPR/Cas9 technology, the major egg white protein has been successfully replaced by recombinant therapeutic proteins and showed efficient accumulation of these therapeutic proteins in the egg white. Recently, we precisely inserted human IgG1 Fc (hIgG1 Fc) coding sequence into the serum albumin (ALB) gene, and generated genome edited chickens that secrete hIgG1 Fc in liver-specifically. The hIgG1 Fc derived from liver circulated bloodstream and eventually accumulated into egg yolk. The liver-derived hIgG1 Fc has N-glycosylation pattern of abundant terminal α-2,6 sialylation and low level of core fucosylation, which is beneficial to inducing anti-inflammatory activity of monomeric hIgG1 Fc. These chicken bioreactor systems employ cutting-edge genetic engineering techniques to integrate desired recombinant protein genes into the chicken genome, effectively transforming the oviduct or liver into a large-scale bioreactor for recombinant protein production. The high-quality recombinant proteins are accumulated in the egg whites and yolks, providing a natural, affordable, and sustainable method. Further research is expected to refine chicken bioreactor system and broaden its application to produce other valuable protein therapeutics.

Transgenic Chickens Re-expressing Evolutionarily Lost Immune Sensors: Spotlight on the Evolution of the Avian Immune System

Hicham Sid[1], Theresa von Heyl[1], Rodrigo Guabiraba[2], Samantha Sives[3], Lonneke Vervelde[3], Sascha Trapp[2], Benjamin Schusser[1]

The retinoic acid-inducible gene I (RIG-I) is a cytoplasmic sensor responsible for the activation of mitochondrial antiviral-signaling proteins (MAVs) and triggering the antiviral response via type I and type III interferons (IFNs) followed by the production of IFN stimulated genes (ISGs). Recent studies indicated that chickens lost this gene throughout evolution with a ubiquitin ligase RIG-I regulator, RNF135. Both of these genes are well conserved in wild ducks, which was speculated to be in association with the immune response of these birds toward influenza.

In this study, we genetically engineered the chicken genome to express duck genes RIG-I and RIPLET/RNF135. We genetically modified chicken primordial germ cells (PGCs) that were re-introduced to chicken embryos raised to sexual maturity to obtain chimeric roosters. Heterozygous chickens expressing both duck genes are healthy and reached sexual maturity with no apparent abnormalities. Furthermore, we monitored the effect of RIG-I expression on the adaptive immune response, including B and T cell populations. Interestingly, RIG-I transgenic chickens had a significantly higher number of T cells than their wild-type siblings ($p<0.05$).

Challenge experiments with a highly virulent avian influenza virus H7N1 showed that the genetically engineered birds developed a stronger inflammatory response than wild-type birds resulting in pronounced clinical symptoms. Our data provide new information about the dynamic evolution of immune sensors in chickens and demonstrate that the RIG-I, an innate immune sensor, can influence adaptive immunity, contributing to the understanding of the role of this gene in chickens as well as wild birds.

[1]TUM School of Life Sciences, Weihenstephan, Department of Molecular Life Sciences, Reproductive Biotechnology, Freising, Germany
[2]Infection and Innate Immunity in Monogastric Livestock, Nouzilly, France
[3]The Roslin Institute & R(D)SVS , University of Edinburgh, UK

Poster Pitches

1. **Mitchell Angove**, UC Davis
   Generation of CD209 gene-edited knockout sheep via electroporation

2. **Kelsey Briggs**, U.S. National Poultry Research Center, USDA-ARS
   Insertion of mouse Mx gene enhances resistance to avian influenza virus in chicken DF1 cells

3. **Dawn Cayabyab**, UC Davis
   A bovine embryonic stem cell reporter line for assessing CRISPR-Cas9 editing efficiency

4. **Jeong Hoon Han**, Pennsylvania State University
   Evaluation of CRISPR activation toolkit for targeted activation of regulatory elements in chickens

5. **Jennifer Jankovitz,** UC Davis
   Androgen Receptor developmental timeline and expression patterns in fetal pigs.

6. **Alba Ledesma**, UC Davis
   Global status of gene edited food animals and their agri/food/feed products

7. **Sebastian Lobo**, UC Davis
   Use of emerging CRISPR/Cas9 technology to improve homology directed repair to generate transgenic pigs.

8. **Jada Nix,** Virginia Tech
   CRISPR-Cas DNA and RNA targeting (DART) for disruption of OCT4 in cattle zygotes.

9. **Theresa Pauli,** Technical University of Munich
   Translational pig models for gastrointestinal diseases

10. **Sabrina Schleibinger**, Technical University of Munich
    Targeting of the chicken serine protease TMPRSS2 in the context of Influenza A virus infection in chickens

11. **Theresa Von Heyl,** Technical University of Munich
    The knockout of αβ T cells causes cytotoxic reactions in chicken – new insights into the functions of T cell subpopulations

12. **Austin Weber**, UC Davis
    Supplementation of human lysozyme goat milk in recipients of standard myeloablative allogenic hematopoietic stem cell transplantation

Session 3: Agricultural Applications I

Chair: Goetz Laible, AgResearch, New Zealand

Production of Genome Edited Pigs Is Supported By Recent Technological Advancements

Kiho Lee, Animal Sciences Research Center, University of Missouri-Columbia

Recent advancements in genome editing technology have lowered the efforts required to establish genome edited pigs. Targeted genetic modifications can be efficiently introduced by 1) genetically engineering the genome of somatic cells to serve as donor cells for somatic cell nuclear transfer (SCNT) and 2) the delivery of genome editing reagents into developing embryos. Improvements in assisted reproductive technologies and genomics tools have also been critical to successfully utilize genome editing systems to produce customized pig models. For example, successful production of in vitro-derived oocytes and embryos is vital to generate genome edited pigs via SCNT or direct editing of the embryonic genome. In addition, various genome analysis tools are available to formulate effective approaches to design genome editing systems that carry high specificity with inconsiderable side effects. While genetically engineered pigs have been widely produced for biomedical applications due to available resources and funding, genome edited pigs tailored for agricultural applications are starting to emerge. Current and future development in genome editing tools and other tools associated with the production of genetically engineered pigs will have a significant effect on the availability of pig models used in agriculture and biomedicine.

Thamani Dairy Cattle

Daniel F. Carlson, Recombinetics/Acceligen

Dairy breeding systems in Africa currently rely on crossbreeding admixed indigenous animals with germplasm from exotic breeds to increase milk yield. However, when the proportion of foreign-derived genetics exceeds the F1 generation, the resulting animals become challenging to manage due to heat stress and susceptibility to endemic diseases. To address these issues, the African Dairy Genetic Gains (ADGG) program has initiated a development program incorporating genomic selection. In this study, we propose augmenting these efforts by creating a battery of Thamani (meaning valuable or precious in Swahili) bulls gene edited to accelerate the genetic gains from the ADGG program.

The primary objective of this investment is to develop two complementary lines of dairy breeds, Holstein and Gir, using elite genetics that can be intercrossed repeatedly without compromising production or adaptive traits. Stakeholders in Sub-Saharan Africa (SSA) were engaged in selecting major effect traits affecting heat tolerance, fertility, trypano-tolerance, resistance to tuberculosis, and increased milk production output for introgression using gene editing technology. Allele candidates for these traits were evaluated, and variants in PRLR (SLICK), FDX2, DHRS4, and MARCO were chosen for introgression into Holstein, and FDX2, DHRS4, ARhGAP15, and GHR for introgression into Gir.

To expedite the process and derive sufficient baseline genetics within a short timeframe, our goal was to produce four multiply edited founders of each sex for Holstein and Gir. To accomplish this, we developed a pipeline for multiplex gene editing and cloning in bovine embryonic stem cells in collaboration with TransOva genetics. Currently, we have produced ten multiplex edited Holstein founders, with ongoing pregnancies in Gir. These animals will support downstream objectives, including

progeny characterization with in-country collaborators, regulatory approval, and determination of economic value before integrating AI hubs and establishing stable breeding programs.

Genetic Engineering of Reproductive Performance in Livestock

Michela Ciccarelli[1,2] and Jon M. Oatley[2]

Use of selective breeding in food animal production is key to trait improvement and addressing current and future challenges in food security. Although assisted reproductive technologies such as artificial insemination and embryo transfer can bolster the impact of selective breeding through increased access to sperm and embryos possessing desirable genomes, their utility is limited to intensive production systems and in some species requires surgical interventions. Thus, there is need for novel reproductive strategies that involve natural mating to achieve widespread and large-scale dissemination of elite trait driving genetics. Spermatogonial stem cell transplantation (SSCT) is one such strategy in which sperm-producing stem cells (aka spermatogonial stem cells or SSCs) are isolated from testes of a select donor male and transferred into the testes of recipient males where engraftment occurs and sperm possessing the donor genome are generated. Translating this methodology from an experimental context to application in livestock production requires recipient males that have testes ablated of endogenous germline but otherwise normal structure and support cell function. To achieve this, the NANOS2 gene has been targeted for inactivation by CRISPR-Cas9 editing in mice, pigs, goats, and cattle. Initial proof-of-concept studies with mice demonstrated that males are germline ablated but attain natural fertility following transplant with wild-type SSCs. In translating this approach to livestock, we discovered that targeted knockout of NANOS2 in pigs, goats, and cattle leads to male-specific sterility due to germline ablation. Similar to mice, testes of NANOS2 knockout livestock were found to be capable of harboring regeneration of donor-derived spermatogenesis following transplantation of donor SSCs. Collectively, these achievements represent key steps in the development of SSCT as a breeding concept in livestock that will be a critical tool for impacting how meat, milk, and fiber are produced to feed an expanding global human population over the coming century.

[1]Departement of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA
[2]School of Molecular Bioscience, College of Veterinary Medicine, Washington State University, Pullman

Session 4: Products to the Market

Chair: Mark Walton, AquaBounty Technologies, USA

Large Scale Generation of Commercially Elite PRRS Virus Resistant Pigs Using CRISPR-Cas

Mark Cigan, Genus Research

Disease resistance genes in livestock provide health benefits to animals and opportunities for farmers to meet the growing demand for inexpensive, high-quality protein. Previously, researchers used gene editing to modify the porcine CD163 gene and demonstrated resistance to the RNA virus that causes a disease in pigs called porcine reproductive and respiratory syndrome (PRRS). To maximize impact, this disease resistance trait needs to exist in commercially relevant breeding populations for multiplication and distribution of pigs. To this end, CRISPR-Cas, next generation DNA sequencing, in vitro and in vivo off-target analyses, WGS and a straightforward breeding strategy were employed to develop a first-of-its-kind, scaled gene editing program to introduce a single modified CD163 allele in four genetically elite and diverse porcine lines which, in cells and animals resist PRRS virus (PRRSV) infection. This founder population, is now being used for large scale phenotype testing, multiplication by classical breeding, continued genetic improvement and ultimately sale upon regulatory approvals. The process and data for this scaled production will be presented.

Methodologies for Setting Up and Conducting On Farm Trails for Practical Data

Caitlin Cooper, Hendrix Genetics

There has been a significant push within the poultry layer industry in the last few years to develop new technologies that eliminate the culling of day old male chicks. In multiple countries in Europe the practice is currently outlawed, and in other regions like North America and Australia there is a strong desire within the industry to phase out the practice. Currently the only methods for sex sorting that have been commercialized involve incubating eggs for multiple days and involve very expensive equipment or invasive sampling techniques. While these technologies are a big step forward, we believe that genetic technologies can be implemented to allow for point of lay sex sorting by placing a detectable marker on one of the sex chromosomes and relying chromosomal segregation to allow for sorting of the population. Significant scientific progress has been made to bring this technology from an idea to an implementable solution. However, to fully realize this goal it must be applied at a considerable scale given the number of layer hens hatched every year. Taking the technology from a laboratory setting into a production setting also poses multiple logistical, regulatory, and market challenges, which often vary from jurisdiction to jurisdiction. As part of advancing this project streamlined and high throughput methods for screening and genetic characterization are being developed. There has also been engagement with regulators in multiple countries to better understand their frameworks with a particular focus on the use of chromosomal segregation. Overall progress has been made on the technical, logistical, and regulatory fronts but there is still a considerable amount of work to be done before this technology can be adopted by the industry.

Application of Genome Editing Technology to Marine Fish Aquaculture

Keitaro Kato[1], Youhei Washio[1], Mitsuki Ohama[2], Kenta Kishimoto[2], Tadanori Umekawa[2], Masato Kinoshita[2,3]

We are engaged in the application of genome editing technology to expedite the breeding process of red sea bream (Pagrus major). Our objective is to enhance the proportion of edible tissue, which currently accounts for less than 40% of the total body weight. To achieve this, we employed the CRISPR-Cas9 system to induce functional disruption of the myostatin gene. Subsequently, in the second generation of genome editing, we successfully generated homozygous mutants. Comparative analysis revealed a 1.2-fold increase in muscle mass in the mutants compared to the wild-type fish. Notably, our feeding trial demonstrated that the mutants exhibited enhanced growth rates even when fed under identical conditions as the wild-type fish, thereby underscoring their potential as a superior aquaculture strain. Presently, the cultivation of genome-edited fish necessitates land-based facilities. To facilitate the commercial production of genome-edited fish, it is imperative to develop economically viable aquaculture technologies, even within high-cost land aquaculture settings. Our ongoing research is dedicated to addressing this requirement.

In Japan, the distribution of genome-edited foods involves three ministries and one agency. The Ministry of the Environment is responsible for establishing criteria to determine whether an organism has been genetically modified. Subsequently, the Ministry of Agriculture, Forestry, and Fisheries (MAFF) evaluates the organism's characteristics, while the Ministry of Health, Labor, and Welfare (MHLW) assesses the organism's safety as a food product. Additionally, the Consumer Affairs Agency is entrusted with defining regulations pertaining to the labeling of genome-edited foods. We have duly completed the necessary prior consultations and notifications with MAFF and MHLW, which have enabled us to commence the distribution of genome-edited fish. Presently, we are actively selling genome-edited fish through crowdfunding platforms and online stores.

[1] Aquaculture Research Institute, Kindai University, 3153, Shirahama, Nishimuro Wakayama, 649-2211 Japan
[2] Regional Fish Institute, Ltd., Yoshida-Honmachi, Sakyo-ku, Kyoto, 606-8501, Japan
[3] Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto, 606-8502, Japan.

Session 5: Regulation - Policy

Chair: Jim Murray, Department of Animal Science, UC Davis

Enabling Regulatory Policies for Animal Biotechnology: A Global Overview

Diane Wray-Cahen, USDA Office of the Chief Scientist

Animal agriculture faces unprecedented challenges. Productivity must increase to meet rising demands for high quality protein, while combating increasing pest and disease pressures, improving animal welfare, and adapting to a changing climate, while also reducing environmental impact. Animal biotechnology has the potential to help address these challenges, but only if global regulatory policies allow for its use in breeding programs and on farms. Access to biotechnology tools and how animal breeders can utilize these tools are dictated by regulatory policies. Regulators are responsible for ensuring a safe food supply, whether produced via conventional breeding methods or biotechnologies. For products of rDNA technologies, approvals are needed before products can enter the marketplace, and the high cost of the approval process limited who sought approval, and for what products. Genome editing has not only changed the way that scientists are introducing DNA changes, but also the way that government agencies are approaching its regulation. The global regulatory landscape for products of genome editing is rapidly evolving. An increasing number of countries are focusing more on characteristics of products and whether they could be achieved by conventional breeding, rather than the technologies used to create them, using risk-proportionate approaches that encourage innovation and allow safe innovations to be used on farms. Unlike regulations for rDNA technologies, many countries are working to develop policies for genome editing that apply to both plant and animal applications. Many of these new policies are based on the definition of living modified organisms in the Cartagena Protocol on Biosafety. There is a growing momentum towards regulatory approaches that encourage innovation to ensure continued access to a safe, abundant, and affordable food supply for today and the future.

Animal Biotechnology and International Trade: Key Considerations

Dan Kovich, USDA Foreign Agricultural Service

International trade in animal products is critical to the economic viability of livestock and poultry industries and global food security. Recent events have highlighted the complexity and inherent fragility of supply chains—and the significant costs associated with their disruption. Farmer enthusiasm for the potential animal biotechnology offers to combat key production challenges is worldwide and growing—but in order to adopt these solutions farmers will need assurance that global markets will accept their products. Market acceptance will require welcoming regulatory environments in trading countries, and a level playing field concerning market conditions such as traceability and labeling. Conventions such as the Cartagena Protocol on Biosafety are key to the former; the latter falls under the auspices of the World Trade Organization (WTO) and its associated bodies. The WTO's primary mechanism for addressing trade in animals and animal products is the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS) which covers food safety and animal health. SPS in turn relies on the Codex Alimentarius Commission (Codex) and the World Organization for Animal Health (OIE) to establish international standards. The WTO's Agreement on Technical Barriers to Trade (TBT) also covers factors such as product labeling. There is currently considerable global discussion concerning the applicability of these agreements to the products of new genetic technologies such as genome editing—but there is no doubt as to their applicability to balancing the routine trade in animal product with national goals to protect food safety and animal health. Developers of animal biotechnologies need to consider how new

innovations may impact the health status of the target animal population or composition of the products it produces as well as its status as a biotechnology in order to anticipate and mitigate potential trade concerns and smooth the way for farmer adoption.

Session 6: Regulation – Journeys 1

Chair: Matt Wheeler, University of Illinois, Urbana-Champaign, USA

Regulating Biotechnology Used for Food: A Perspective on Policy from Inside FDA and from the Startup Community

Eric Schulze, UPSIDE Foods

Federal scientific regulation and policy is often perceived as an example of science being a mere observer in the realm of law. However, in the field of biotechnology regulation in the U.S., we are witnessing a different reality. New biotechnologies are successfully navigating regulatory obstacles, allowing for rapid development, deployment, and market entry by various firms. In this session led by Eric Schulze, UPSIDE Foods' VP Global Regulatory and Public Policy, we will explore the UPSIDE model, which outlines effective biotechnology policy regulation.

During the presentation, we will discuss how the UPSIDE model effectively serves all firms in our field, even when diverse production methods are safeguarded as trade secrets. This model exemplifies industry-led initiatives that lead to the creation of new policies, highlighting the importance of genuine partnerships in shaping regulations. Moreover, the model's effectiveness remains consistent across different administrations. Drawing on his experience as a former developer of novel food and drug policy at the FDA, Schulze will provide insights into the successes, challenges, and ongoing efforts to enhance federal regulatory policies through the go-to-market strategy of the world's first cultivated meat product in the United States.

With firsthand experience in obtaining the first-ever approvals for genetically-engineered animals, Schulze offers a unique perspective during the presentation. He will emphasize the shift in policy-making approach when transitioning from federal service to UPSIDE Foods, emphasizing a commitment to shaping policy for the benefit of the entire industry. By sharing insights from successful cultivated meat safety consultations at the FDA and the label approval process at the USDA, Schulze aims to inspire the exploration of new and more efficient pathways for introducing genetically-engineered animal products to the market.

Sterility is a Foundational Trait for the Large-Scale Commercialization of Genome Editing and Genetic Biotechnologies for Aquatic Species

John T. Buchanan, Center for Aquaculture Technologies, USA

Aquaculture is growing to meet the needs of an increasing world population, and aquaculture genetics must move quickly to help producers meet the expectations of consumers and retailers. Genome editing offers the opportunity to make targeted changes in the genome, introducing variation that will result in rapid and substantial improvements in performance, health and sustainability. To responsibly introduce fish which harness the power of genetic technologies into commercial production systems, the farmed fish should be sterile or otherwise contained. The ability to produce sterile progeny from broodstock for aquaculture also has significant benefits to productivity and environmental sustainability. We describe the development of a strategy to generate, breed and mass-produce sterile fish.

Our strategy for mass producing sterile fish is designed to produce monosex, sterile populations. We first investigated gene mutations in two evolutionarily conserved pathways, one governing sex differentiation and the other sexual competency. We created edits in genes necessary for spermiogenesis and steroid hormone synthesis causing male sterility and masculinization, respectively. Double gene edit combinations for these genes produced all-male sterile populations. Likewise, we created variants in genes whose inactivation caused females to develop arrested or string-like ovaries lacking oocytes. We further disrupted genes causing genetic males to develop as females. Double gene edit combinations for these genes produced all-female, sterile populations.

Propagation of the double KO lines was achieved via germ cell transplantation from a juvenile edited donor into a germ cell free wild-type recipient embryo. In the resulting recipients, the induced edits had no effect as the genes targeted are not expressed in germ cells. With this approach, we generated fertile broodstock that successfully mass-produced sterile, monosex populations. We expect that adoption of this technology will result in broad economic and environmental benefits for aquaculture, and as a foundation upon which to build additional genetic technologies.

Session 7: Regulation – Journeys 2

Chairs: Simon Lillico, The Roslin Institute, United Kingdom

Working in the Regulated Environment in the US, Canada and Internationally: AquaBounty's Experience

Chantal March, AquaBounty, Canada

The original founder of AquAdvantage salmon was created from one single micro-injection event over thirty years ago. People often cite the time it took AquaBounty to successfully achieve regulatory approval and launch commercial production as a barrier to developing genetically engineered (GE) animals. The regulatory process can be daunting. However, unlike the mid-1990s when AquaBounty's predecessors began their regulatory journey, the process in the United States and Canada is more clearly defined.

Innovators themselves must take responsibility for their success in the regulatory process. Clearly defining the product that will be offered to consumers is the most important step. Innovators must then be prepared to demonstrate that their product performs as expected, animal welfare is ensured, environmental risk is mitigated, and that the final product is safe for humans.

Working and communicating with regulators and the public is key. In some regulatory jurisdictions, clear regulations may still not be in place for GE animals. Regulators may be inclined to adapt their current regulations based on existing knowledge which may not be appropriate. It becomes incumbent on innovators to understand what is being requested by regulators and be prepared to question the need for the requested data if necessary. Education of the regulators may be key to your regulatory success. Regulators must also be encouraged to utilize the tools of today's rapidly evolving technology to assess the risks of the GE animal, thereby lessening the cost of regulatory burden. For companies with an approved product who consistently demonstrate a commitment to environmental stewardship, identifying ways to lessen the regulatory burden is key to commercial expansion.

AquaBounty is also looking to the future with the addition of gene editing into our product pipeline. Being able to successfully utilize the technology to address everyday issues in the aquaculture industry

will require continued dialogue with regulators. This will be critical to ensuring such innovations can be used to their full potential.

PRRSV-resistant pigs: Genus PLC Global Regulatory Strategy and Experience

Clint Nesbitt, Genus, USA

Genus PLC, a global animal genetics company focusing on improvement of beef, dairy, and pork, has used a CRISPR-based approach to develop multiple lines of pigs resistant to the virus causing Porcine Reproductive and Respiratory Syndrome (PRRS). In this presentation, we will provide an overview of Genus' global regulatory strategy, experience working with regulatory agencies to date, and the interplay between regulatory approvals and commercialization on a global scale.

GalSafe™ Pig Regulatory Journey

John Bianchi, A. Walters, & D. Ayares, Revivicor, Inc, Blacksburg VA

After a rigorous regulatory review process, the GalSafe™ pig with an intentional genomic alteration (IGA) has been approved by the Food and Drug Administration (FDA) for food and potential human therapeutic use. The objective of this presentation is to provide background on the IGA; GalSafe™ pigs do not have detectable galactose-α1,3-galactose (α-gal) on cells, tissues, or organs. Further, the purpose and use of derivatives from GalSafe™ pigs for food and therapeutic use will be described. Importantly, the presentation will highlight each discrete step in the risk-based hierarchical approach established by the FDA-Center for Veterinary Medicine (FDA-CVM) that ultimately led to FDA approval. The FDA-CVM review began with the product definition and proceeded through a step-wise series of investigations to characterize the potential hazards associated with the rDNA construct, the lineage of the animal with the IGA, and the durability of its genotype and phenotype. In addition, the regulatory review included sections dedicated to food and feed safety assessment as well as an environmental assessment.

Session 8: Agricultural Applications II

Chair: Anna Denicol, University of California, Davis, USA

First Gene-edited Calf With Reduced Susceptibility To A Major Viral Pathogen

Aspen M Workman[1], Michael P Heaton[1], Brian L Vander Ley[2], Dennis A Webster[3], Luke Sherry[3], Sabreena Larson[4], Theodore S Kalbfleisch[5], Gregory P Harhay[1], Erin E Jobman[2], Daniel F Carlson[3], Tad S Sonstegard[4]

Bovine viral diarrhea virus (BVDV) is one of the most important viruses causing respiratory and gastrointestinal diseases and reproductive failure in cattle throughout the world. The main BVDV cellular receptor in cattle is CD46 and residues G82QVLAL87 are among those essential for virus binding and entry. Substituting residues A82LPTFS87 in CD46 was previously shown to reduce BVDV susceptibility in vitro. Here we used CRISPR-mediated homology-directed repair and somatic cell nuclear transfer to produce a live calf with homozygous CD46 A82LPTFS87 substitutions. The result was a gene-edited calf with dramatically reduced susceptibility to infection as measured by reduced clinical signs and the lack of viral infection in white blood cells. The edited calf has no off-target edits and appears normal and healthy at 23 months of age without obvious adverse effects from the on-target edit. This precision bred, proof-of-concept animal provides the first evidence that intentional genome alterations in CD46 may reduce the burden of BVDV-associated diseases in cattle, and is consistent with our stepwise, in vitro and ex vivo experiments with cell lines and matched fetal clones.

[1]US Meat Animal Research Center, United States Department of Agriculture (USDA), Agricultural Research Service (ARS), Clay Center, NE, 68933, USA
[2]University of Nebraska-Lincoln, Great Plains Veterinary Educational Center, Clay Center, Nebraska, 68933, USA
[3]Recombinetics Inc., Eagan, MN, 55121, USA
[4]Acceligen Inc., Eagan, MN, 55121, USA
[5]Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, KY, 40506, USA

Production and Germline Transmission of MSTN Gene Edited Cattle

*Gyeong-Min Gim[1,2], Goo Jang[1, 2]**

In the fields of agriculture, genome editing technologies including clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 have long been recognized as a potent tool for increasing productivity and deciphering breed-specific genetic regulation. However, there are few guidelines for the application of this technique to cattle. The goal of this study was to produce trait-improved cattle using the genome-editing technology CRISPR–Cas9 and confirm the germline transmission of the mutation into next generation (F1). Myostatin (MSTN) was selected as a target locus, and synthetic mRNA of sgRNA and Cas9 were microinjected into in vitro fertilized bovine embryos. Blastocysts were transferred to surrogate mothers and knockouts were analyzed using T7E1 assay in the calves. Gene-edited calves were raised to study the germline transmission of the mutation to the next generation. The founder's (F0) germ cells were isolated, fertilized with wild type germ cells, and transferred into the recipients. As a result, 17 healthy calves were born, and three of them showed MSTN mutation rates of 10.5%, 45.4%, and 99.9%, respectively. Importantly, the offspring with the 99.9% MSTN mutation rate had a biallelic mutation (-12 bps) and an out growing muscling phenotype. Embryos produced by in vitro fertilization with MSTN male and female cattle were transferred to a surrogate, and three calves were successfully born. MSTN heterozygous mutation was shown by sequencing of the F1 calves, which had no health issues. These data demonstrate that the cattle produced through genome editing matured without health issues and had transmitted MSTN mutation from the germ cells. These genome editing resources could be used to develop a new breed of lean meat in the future.

[1]Laboratory of Theriogenology and Biotechnology, Department of Veterinary Clinical Science, College of Veterinary Medicine, the Research Institute of Veterinary Science, and BK21 Four Future Veterinary Medicine Leading Education & Research Center, Seoul National University, Seoul, Republic of Korea [2]LARTBio Inc., Seoul, Republic of Korea

Improving Disease Resistance in Aquaculture: Integration of Genome Editing into Breeding Programs

Diego Robledo, The Roslin Institute, United Kingdom

Aquaculture is the fastest growing food production sector, and will play a pivotal role in meeting global food demands over the coming decades. However, the growth and sustainability of aquaculture is severely threatened by infectious diseases, which can cause mass mortalities and reduce the growth and welfare of farmed animals. There is a worrying lack of prevention and therapeutic strategies, and the development of vaccines or drugs is infrequent due to the large costs involved. Selective breeding can lead to a decrease in the severity and frequency of pathogen outbreaks, however several generations of selection are required to obtain meaningful improvements, and only a few traits can be improved

simultaneously. In this scenario, the incorporation of genome editing to breeding programmes offers the perfect complement to enhance disease resistance in aquaculture stocks. In this talk, I will describe our efforts to optimize both in vitro and in vivo genome editing in aquaculture species, and how we are using our existing models to find and validate gene candidates to improve resistance to various diseases in aquaculture species.

Session 9: Biomedical Applications

Chair: Kristin Whitworth, University of Missouri, USA

Humanized Minipigs to Assess Drug Safety

Tatiana Flisikowska[1], Jerome Egli[2], Krzysztof Flisikowski[1], Eckhard Wolf[3], Antonio Iglesias[2], Angelika Schnieke[1]

Therapeutic antibodies have significantly improved treatment for various diseases like autoimmune disorders, infectious diseases, and targeted cancer therapy. However, certain recombinant antibodies can cause severe adverse effects that are hard to anticipate. Pre-clinical testing on animals is challenging because they recognize human proteins as foreign, making it difficult to predict immune reactions to therapeutic proteins unless it sees the human protein as self.

Researchers have developed transgenic mouse models expressing human transgenes, but their limited genetic diversity and immune mechanism differences with humans restrict their use for immunogenicity assessment. Additionally, the size of mice makes them unsuitable for certain routes of application, like intravitreal dosing.

Pigs, due to their similarity to humans, are emerging as a better model than rodents and could potentially replace non-human primates (NHPs) for pre-clinical studies. To create a universal pig model for safety testing of human recombinant antibodies, we've generated a transgenic minipigs with a mini-repertoire of human immunoglobulin genes *(IgG1* and *IgG4*). They demonstrate protein production and tolerance to specific human monoclonal antibodies used in clinics[1].

These minipigs are especially valuable as they can predict antibody-dependent immune reactions (ADAs) in humans for certain therapeutic antibodies used in cancer treatment, proving the model's validity for safety assessment and predicting side effects.

Moreover, porcine models closely mirror human pathology in diseases like colorectal cancer[2], osteosarcoma[3], and inflammatory bowel disease[4]. Combining the humanized minipig with these disease models offers an efficient and safe way to assess both the safety and efficacy of novel therapeutic antibodies for cancer treatment.

In summary, using humanized minipigs as an alternative to NHPs provides a representative and predictive platform for pre-clinical safety testing of therapeutic antibodies. When combined with disease models, it becomes a valuable tool to bring new therapeutics to the clinic efficiently and safely.

[1]Chair of Livestock Biotechnology, School of Life Sciences, Technical University München, Freising, Germany, [2] Roche Pharmaceutical Research and Early Development, Roche Innovation Center Basel, Basel, Switzerland, [3] Gene Center and Department of Veterinary Sciences, Molecular Animal Breeding and Biotechnology, Ludwig-Maximilians-Universität München, Munich, Germany

1. Flisikowska T., Egli, J., Flisikowski, K., Stumbaum M., Küng E., Ebeling M., Schmucki R., Georges G., Singer T., Kurome M., Kessler B., Zkhartchenko V., Wolf E., Weber F., Schnieke A., Iglesias A. (2022). A humanised minipig model for the toxicological testing of therapeutic recombinant antibodies. *Nat. Biomed. Eng*. 6, 1248–1256.
2. Flisikowska T, Merkl C, Landmann M, Eser S, Rezaei N, Cui X, Kurome M, Zakhartchenko V, Kessler B, Wieland H, Rottmann O, Schmid RM, Schneider G, Kind A, Wolf E, Saur D, Schnieke A. (2012). A porcine model of familial adenomatous polyposis. *Gastroenterology*. 143(5):1173-1175.e7.
3. Saalfrank A, Janssen KP, Ravon M, Flisikowski K, Eser S, Steiger K, Flisikowska T, Müller-Fliedner P, Schulze É, Brönner C, Gnann A, Kappe E, Böhm B, Schade B, Certa U, Saur D, Esposito I, Kind A, Schnieke A. (2016). A porcine model of osteosarcoma. *Oncogenesis*. 14;5(3):e210.
4. Winogrodzki T., Metwaly A., Grodziecki A, Liang W, Klinger B., Flisikowska T., Fischer K., Flisikowski K., Steiger K., Haller D., Schnieke A. (2023). *TNF$^{\Delta ARE}$* pigs: a translational Crohn's Disease model. *J. Crohns Colitis.* 23:jjad034.

Humanized Skeletal Muscle: Novel Models and Therapies

Mary Garry, Integrative Biology and Physiology, Medical School, University of Minnesota

Volumetric muscle loss (VML) occurs when the native elements required for muscle regeneration and repair are lost. Volumetric muscle loss typically results in persistent strength and functional deficits and long term disabilities. Current therapies for VML, due to disease or injury, are largely ineffective. Typically, autologous muscle grafts are used for reconstruction but often result in donor site morbidity. Synthetic and biologic scaffolds result in fibrosis and poor functional outcome and, therefore, new reconstructive strategies are warranted. Our goal is to engineer human skeletal muscle in pigs for use in transplantation. To do that, we cloned multiplex gene edited porcine fibroblasts (MYF5/MYOD/MYF6 deletions) to create skeletal muscle null porcine embryos. We confirmed that these embryos lack the ability to produce skeletal muscle and, thus, provide a non-competitive niche for exogenous cells to populate. To replace the absent skeletal muscle in these null embryos, we used blastocyst complementation to deliver exogenous cells to produce either intra- or inter-species chimeric animals. As proof of feasibility, we demonstrated that the intraspecies (pig:pig) chimeras were viable and displayed normal histology, morphology, and function. We have also engineered interspecies (human:pig) chimera with enhanced chimerism capabilities and confirmed the restoration of muscle markers in the null porcine embryo using immunohistochemical and molecular techniques. These data support the feasibility of generating human:pig chimeras and provide a platform for the purpose of generating exogenic organ production to serve as a future organ source for transplantation following VML injuries.

Development and Characterization of a Novel Sickle Cell Disease Sheep Model

Irina A. Polejaeva[1], Iuri V. Perisse[1], Caroline E. Kuczynski[2], Ying Liu[1], Christopher D. Porada[2], Graça Almeida-Porada[2]

Sickle cell disease (SCD) is the most common inherited blood disorder in the US caused by a missense mutation in the *HBB* gene, which encodes the β-subunit of hemoglobin (Hb). The sheep β-globin gene shares approximately 87.5% identity with the human gene, and sheep hematopoietic development parallels that of humans including a developmental γ- to β-globin switch, making sheep an ideal model to provide insights into the pathobiology of SCD or to test much needed novel therapies. We introduced the SCD mutation in sheep fetal fibroblasts (SFFs) using CRISPR/Cas9 Homology Directed Repair (HDR) gene editing approach and subsequently used these SFFs for the production of SCD lambs by somatic

cell nuclear transfer (SCNT). We transferred 104 SCD cloned embryos into 8 estrus synchronized recipients. Three pregnancies (3/8=37.5%) were confirmed by ultrasonography around day 40 of gestation. All pregnancies went to term and gave birth to 3 SCD lambs. Blood from SCD lambs, and age-matched wildtype controls (WT) were evaluated by the same diagnostic methods used in humans: hematocrit, blood smears, reticulocyte counts, alkaline and capillary Hb electrophoresis, and Hb solubility tests. Blood smears demonstrated the presence of sickled cells, acanthocytes, target cells, Howell-Jolly bodies, normoblasts, and polychromasia typical of SCD, while smears from WT animals were normal. Alkaline electrophoresis demonstrated the presence of Hb with the same migration pattern as human HbS in SCD animals, which was not present in WT, and capillary Hb electrophoresis confirmed the presence of the abnormal Hb in SCD sheep. The presence of a putative HbF band was also identified, decreasing from birth to day 24. Furthermore, the Hb solubility test confirmed the presence of HbS in SCD, whereas it was negative in WT controls. Additionally, we recently generated a group of heterozygote lambs (n=12) by breeding of WT ewes to a homozygous SCD (HbS/HbS) ram. These animals will be used for future SCD sheep line expansion and model characterization. Our data indicate that introduction of the human SCD mutation in sheep genome results in a similar phenotype at the blood level, thereby potentially producing a similar disease phenotype and progression in the sheep model. Our findings therefore support the translational relevance of our SCD sheep model of its potential applications in the development of novel therapies for SCD.

This work was supported in part by the Utah Agricultural Experiment Station (project 1742) at Utah State University, the USDA/NIFA multistate research project W-4171 and with Federal funds from the Intramural Research Program of the National Center for Advancing Translational Sciences, National Institutes of Health under Contract No. 75N95023P00054.

[1]Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, UT, USA
[2]Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston-Salem, NC, USA

Closing: Summary and Future

Eric Hallerman, Virginia Tech University

Poster Abstracts

A

Heavy chain-only transgenic chickens producing human antibodies demonstrate robust immune repertoires and high-affinity binding

Christine N. Vuong, Kevin M. Reynolds, Abheepsa Gupta, Baisen Zeng, Philip A. Leighton

OmniAb, Inc. Emeryville, CA, USA

Heavy chain-only immunoglobulins are naturally produced in camelids and sharks. Because the structure does not utilize light chain, the antigen-binding region (VH of heavy chain-only, or VHH) is half the size of a traditional antibody, allowing for unique interactions with antigenic epitopes. Chicken immunoglobulins have a standard heterodimeric structure comprised of both heavy and light chains, and do not naturally include VHH forms. We have developed an engineered chicken that produces VHH antibodies with human variable regions. In these birds, the human VH contains select framework mutations to provide stability and a truncated light chain that facilitates immunoglobulin secretion in the absence of the VL domain. Productive B-cell development is observed in these transgenic birds, which express normal levels of serum IgM and reduced levels of IgY. When three different targets were immunized into the VHH chickens, robust immune responses were observed. Recovered antigen-specific VHH antibodies offered a diverse repertoire of VHH sequences, broad epitope coverage, and binding affinities reaching single-digit nM KD.

B

The knockout of αβ T cells causes cytotoxic reactions in chicken – new insights into the functions of T cell subpopulations

Theresa von Heyl, Romina Klinger, Dorothea Aumann, Christian Zenner, Mohanned Alhussien, Antonina Schlickenrieder, Kamila Lengyel, Hanna-Kaisa Vikkula, Teresa Mittermair, Hicham Sid, Benjamin Schusser
Biotechnology of Reproduction, TUM, Freising, Germany

So far, no animal model has been developed to study T cell subpopulations in chicken. To investigate the role of $\alpha\beta$ and $\chi\delta$ T cells in birds, we generated chickens lacking these T cell populations. This was achieved by genomic deletion of the constant region of the T cell receptor β or χ chain, leading to a complete loss of either $\alpha\beta$ or $\chi\delta$ T cells. Surprisingly our results showed that a deletion of $\alpha\beta$ T cells but not $\chi\delta$ T cells resulted in a severe phenotype in chicken, as demonstrated by granulomas inflammatory reactions in the spleen and the proventriculus. The immunophenotyping revealed a significant increase in monocytes and the absence of CD4+ T cells and FoxP3+ regulatory T cells compared to wild type chickens. In the $\alpha\beta$ T cell knockout chickens, we observed a significant decrease in immunoglobulins, B lymphocytes, and changes in the bursa morphology. Our data reveals for the first time the consequences of T cell knockouts in chickens and provide new insights into avian T cell functions.

C

Insertion of mouse Mx gene enhances resistance to avian influenza virus in chicken DF1 cells

Kelsey Briggs, Ryan, Sweeney, Klaudia Chrzastek, Karen Segovia, Darrell R. Kapczynski

U.S. National Poultry Research Center, USDA-ARS

Avian influenza virus (AIV) is a highly contagious and lethal disease that can have major impacts on the global poultry industry and food supply. In recent years, the U.S. has seen an increase in the incidence of highly pathogenic avian influenza virus (HPAIV) outbreaks in wild birds and commercial poultry. In 2014/2015 and in 2021/2022 outbreaks of HPAIV subtype H5NX clade 2.3.4.4 resulted in the death and destruction of over 50 million birds each costing billions of dollars to the U.S. economy. Vaccines are not currently approved for use in the U.S., so control strategies for HPAIV are dependent on biosecurity and culling of infected flocks. New strategies for HPAIV control based of gene editing of poultry species could offer solutions for disease control. Mx proteins are GTPases that directly interact with the AIV nucleoprotein. In mice, the Mx1 gene is a critical determinant of resistance to influenza virus. Chickens have a single Mx gene, but there are conflicting reports on the effectiveness of the chicken protein against AIV. Here we demonstrate a strategy for improving disease resistance to HPAIV based on enhancing host innate immunity through the addition of mouse Mx1 gene using a miniTol2 transposon system. The in vitro results demonstrate reduced virus titers and cytopathic effect (CPE) following HPAIV and LPAIV infection. These results demonstrate that mouse Mx could be useful for enhanced protection of AIV in birds.

D

Targeting of the chicken serine protease TMPRSS2 in the context of Influenza A virus infection in chickens

Sabrina Teresa Schleibinger, Benjamin Schusser, Hicham Sid, TUM School of Life Sciences, Weihenstephan, Department of Molecular Life Sciences, Reproductive Biotechnology, Freising, Germany

Influenza A viruses (IAVs) pose a major threat for humans and animals. Cleavage of the influenza hemagglutinin (HA) by serine host proteases is an essential step for viral replication. In case of low pathogenic influenza viruses, the transmembrane protease serine S1 subtype 2 (TMPRSS2) is the major HA-activating protease and represents a promising target in the fight against influenza infections. One possibility to avoid the HA cleavage is the generation of TMPRSS2-/- knock-out (KO) birds missing the functional active serine protease. Therefore, we established a CRISPR/Cas9 based targeting strategy that removes exon 10 to 13 which encodes the serine protease domain, in chicken primordial germ cells (PGCs). The genetically modified PGCs were used to generate chimeric chicken whose sperm samples were tested positive for a KO in the TMPRSS2 serine protease domain. Another way to target host proteases is to use serine protease inhibitors. Therefore, we investigated the suitability of tracheal organ cultures (TOCs) for testing antiviral therapies. We examined the effect of two different protease inhibitors on IAV replication using TOCs. TOCs were divided into different groups, either untreated or treated with aprotinin or camostat mesylate, or both, and subsequently infected with the low pathogenic strains H9N2 or H6N8. We investigated several parameters, including virus replication and loss of ciliary activity (ciliostatis). Our results showed differences in the inhibitory effects of aprotinin

and camostat mesylate. The latter significantly reduced viral replication ($p<0.05$) and delayed the loss of ciliary activity, which was not observed with aprotinin. Our results suggest that TMPRSS2 is a promising target for limiting influenza infection. They also indicate the usefulness of TOCs in comparative drug studies and promise further applications of organ cultures including evaluation of antiviral drug efficacy.

Keywords: proteases, genetically modified chicken, TMPRSS2, knock-out, tracheal organ cultures, avian influenza virus

E

Decreased antiviral response in duck fibroblast after ablation of the RIG-I gene

Bethany K. Redel[1], Lei Shi[2,3], Rachel W. Read[4], Kevin D. Wells[4], Wenjun Ma[2,3], Paula R. Chen[1]
Plant Genetics Research Unit, USDA-ARS, Columbia, Missouri, USA[1]; Department of Veterinary Pathology, University of Missouri, Columbia, Missouri, USA[2]; Department of Molecular Microbiology and Immunology, University of Missouri, Columbia, Missouri, USA[3]; Division of Animal Sciences, University of Missouri, Columbia, Missouri, USA[4]

Recently, outbreaks of highly pathogenic avian influenza virus (AIV) have gravely impacted poultry industries worldwide, as chickens develop severe symptoms which are usually accompanied by high rates of mortality. On the other hand, ducks and other waterfowl serve as natural reservoirs for AIVs and typically display mild symptoms of infection. Contributing to the innate immune response, retinoic acid-inducible gene I (RIG-I) is an RNA helicase that senses short viral double-stranded RNAs within the cytoplasm to induce the production of type I interferons. Phylogenetic analyses have revealed that the RIG-I gene is present in the genomes of most species, including ducks, but is missing in the chicken genome. Thus, absence of RIG-I may partially explain the higher pathogenicity of AIVs in chickens. The objective of the current study is to determine the effect of disrupting the RIG-I gene in duck fibroblasts on their antiviral response. Pekin duck fibroblasts were transfected with the CRISPR/Cas9 system targeting exon 3 of the RIG-I gene, and four predicted knockout colonies with different genotypes were obtained. Wild type and RIG-I knockout cells were stimulated with low or high molecular weight poly(I:C) for 6 or 24 h, and cells were collected for RNA extraction and qPCR analysis to detect transcriptional differences related to antiviral response. Compared to wild type cells, RIG-I knockout cells demonstrated decreased abundance of interferon regulatory factor 7 (IRF7) and radical S-adenosyl methionine domain containing 2 (RSAD2) when stimulated with low molecular weight poly(I:C). Additionally, wild type and RIG-I knockout cells were infected with a lowly pathogenic H9N2 AIV, and no differences in viral titers were observed up to 60 h post-infection. However, abundance of transcripts related to interferon signaling were decreased in RIG-I knockout cells. Overall, knockout of RIG-I in duck fibroblasts suppressed the antiviral response after poly(I:C) stimulation and H9N2 virus infection.

F

Evaluation of CRISPR activation toolkit for targeted activation of regulatory elements in chickens

Jeong Hoon Han and Tae Hyun Kim The Pennslyvania State University, USA

Genome-wide significant variants primarily reside in non-coding regions, necessitating the validation of predicted functional regulatory elements. These regulatory elements exert crucial control over the precise timing, spatial localization, and magnitude of gene expression. The emergence of engineered CRISPR technology serves as a potent tool for functional studies on non-coding regulatory elements in genomes. In this study, we utilized epigenetic data from the Functional Annotation of Animal Genomes (FAANG) to identify putative promoter and enhancer regions of three genes (HBBA, IRF7, and PPARG) in the chicken genome based on DNase hypersensitivity sites (DHS) from lung, spleen, and adipose, respectively. To activate the putative promoters and enhancers of these genes, we designed gRNAs and employed CRISPR activation (CRISPRa) using dCas9-p300 and dCas9-VPR as transcriptional activators in chicken DF1 fibroblast line. In HBBA promoter activation, dCas9-VPR displayed a 3.47-fold increase, and dCas9-p300 demonstrated a 7.76-fold increase compared to the mock. Furthermore, co-transfection of promoter and enhancer targeting gRNAs led to augmented upregulation, with dCas9-VPR achieving a 7.09-fold increase and dCas9-p300 demonstrating a 25.1-fold increase compared to the mock. These values correspond to 2-fold and 3.2-fold increases, respectively, over promoter activation alone. Similarly, in IRF7, while the promoter alone exhibited a substantial 22.1-fold increase compared to the mock, co-activation of promoter and enhancers resulted in a significantly higher 35.7-fold increase in expression compared to the mock (1.62 -fold vs. promoter alone). However, co-activating putative enhancer regions did not result in additional upregulation of PPARG expression, warranting further investigation. In summary, our findings demonstrate the high efficiency of CRISPRa in precisely manipulating endogenous gene expression by targeting regulatory elements in chicken, highlighting its potential for functional validation of non-coding regions such as promoters and enhancers in the chicken genome.

G

Transgenic expression of ACE2 and TMPRSS2 genes from different animal species confers susceptibility to SARS-CoV-2 infection in non-permissive avian cell line

Darrell R. Kapczynski, R. Sweeney, K. Briggs, E. Spackman, D.L. Suarez U.S. National Poultry Research Laboratory, ARS, USDA, Athens, GA, USA

The SARS-CoV-2 (SC2) virus is responsible for a worldwide pandemic because of the virus's ability to transmit efficiently human-to-human. A key determinant of infection is the viral attachment spike protein to the host receptor angiotensin-converting enzyme 2 (ACE2) protein. Because of the presumed zoonotic origin of SC2, there is no practical way to test every species susceptibility to SC2 by direct challenge studies. In an effort to have a better predictive model of animal host susceptibility to SC2, we expressed the ACE2 and/or transmembrane serine protease 2 (TMPRSS2) genes from humans and other animal species in the avian fibroblast cell line, DF1, that is not permissive to SC2 infection. We demonstrated that expression of both human ACE2 and TMPRSS2 genes is necessary to support SC2

infection and replication in DF1 and a non-permissive sub-lineage of MDCK cells. Titers of SC2 in these cell lines were comparable to those observed in control Vero cells. To further test the model, we developed seven additional transgenic cell lines expressing the ACE2 and TMPRSS2 derived from Felis (cat), Equus (horse), Sus (pig), Capra (goat), Mesocricetus (Golden hamster), Myotis lucifugus (Little Brown bat) and Hipposideros armiger (Great Roundleaf bat) in DF1 cells. Results demonstrate permissive replication of SC2 in cat, Golden hamster, and goat species, but not pig or horse, which correlated with the results of reported challenge studies. The use of transgenics allows for more efficient testing of the potential susceptibility of many different animal species for SC2 and emerging variant viruses.

H

Translational pig models for gastrointestinal diseases

Theresa M. Pauli, Laura Beltran Sangüesa, Thomas Winogrodzki, Wei Liang, Daniela Kalla, Krzysztof Flisikowski, Angelika Schnieke, Tatiana Flisikowska, Livestock Biotechnology, Department of Molecular Life Sciences, School of Life Sciences, Weihenstephan, Technical University of Munich, 85354 Freising, Germany

Bridging the gap between preclinical studies and human trials poses a significant challenge. Our group is dedicated to overcoming the difficulties of clinical translation by utilizing genetically engineered (GE) pigs for studying human gastrointestinal (GI) diseases, such as colorectal and pancreatic cancers (oncopigs), as well as Crohn's disease (CD).

GE pigs carrying a nonsense mutation in APC (APC1311) are a valuable model for the predisposition of colorectal cancer (CRC). They develop polyps in the colon resembling the human disease and have been instrumental in identifying novel genetic factors impacting CRC severity. They have also contributed to studying the role of the microbiome in polyp progression and regression. Additionally, the APC1311 pigs have aided in evaluating advanced diagnostic techniques like fluorescent molecular probes, employing artificial intelligence, and endoscopic imaging for early detection.

We generated pigs with endogenous latent KRASG12D and TP53R167H mutations, activatable by Cre, to create a physiologically relevant model for pancreatic ductal adenocarcinoma (PDAC). Additionally, PTF1A and PDX-1 Cre-driver pigs were developed to activate both mutant alleles specifically in the pancreas. Our findings demonstrate that pancreatic expression of KRASG12D, alone or in combination with TP53R167H, induced the development of metastatic PDAC in pigs, replicating key features of the human disease. The pig model offers a unique advantage of longitudinal, multi-regional tumor sampling, facilitating early detection and monitoring of tumor growth and progression. Based on the TNFΔARE/+ mouse, we developed the first porcine CD model, which exhibits remarkable similarity to the human disease, displaying characteristics such as ileocolitis, dysbiotic microbial communities, and immune cell infiltration. To evaluate the model's validity, we conducted treatments using two anti-TNF drugs commonly administered to human patients.

To refine our porcine models and facilitate the generation of new models, we have also generated pigs with ubiquitous expression of Cas9 for in vivo gene editing.

I

Human proinsulin and insulin production in the milk of transgenic cattle

Paulo S. Monzani[1,6], Juliano R. Sangalli[2], Rafael V. Sampaio[2], Samuel Guemra[1], Renato Zanin[3], Paulo R. Adona[1], Maria A. Berlingieri[1], Luiz F.C. Cunha-Filho[1], Irma Y. Mora- Ocampo[4], Carlos P. Pirovani[4], Flávio V. Meirelles[2], Otavio M. Ohashi[5], Matthew B. Wheeler[6]

[1] University of Northern Paraná, Londrina, PR, Brazil, [2] University of São Paulo, Pirassununga, SP, Brazil, [3] Laffranchi Agriculture, Tamarana, PR, Brazil, [4] State University of Santa Cruz (UESC), Ilhéus, BA, Brazil, [5] Federal University of Pará, Belém, PA, Brazil, [6] University of Illinois at Urbana-Champaign, Urbana, IL, USA.

The worldwide growing demand for human insulin for treating diabetes could be supplied by transgenic animals producing insulin in their milk. Pseudo-lentivirus containing the bovine β-casein promoter and human insulin sequences was used to produce modified adult fibroblasts, and the cells were used for nuclear transfer. Transgenic embryos were transferred to recipient cows, and one pregnancy was produced. Recombinant protein in milk was evaluated using western blotting and mass spectrometry. One transgenic cow was generated, and in milk analysis, two bands were observed in western blotting with a molecular mass corresponding to the proinsulin and insulin. The mass spectrometry analysis showed the presence of human insulin more than proinsulin in the milk, and it identified proteases in the transgenic milk that could convert proinsulin into insulin and insulin-degrading enzymes that could degrade the recombinant protein. The methodologies used for generating the transgenic cow were efficient in producing recombinant protein in the milk, and the mass spectrometry for detecting recombinant protein in integral milk. Milk proteases could act on protein processing converting recombinant protein to functional protein. On the other hand, some milk proteases could act in degrading the recombinant protein.

J

Supplementation of human lysozyme goat milk in recipients of standard myeloablative allogeneic hematopoietic stem cell transplantation

Austin Weber[1], Karamjeet Sandhu[2] and Elizabeth A. Maga[3]
[1]Department of Food Science and Technology, University of California, Davis, USA
[2]City of Hope National Cancer Center, Duarte, CA, USA
[3]Department of Animal Science, Uversity of California, Davis, USA

Hematopoietic cell transplantation (HCT) is a stem cell therapy used to treat patients with leukemia and lymphoma that are unresponsive to conventional chemotherapy. HCT therapy involves the depletion of host native bone marrow populations, and an infusion of donor hematopoietic stem cells. A frequent patient complication of HCT is graft-versus-host disease (GVHD), a systemic immune-driven disorder characterized by severe gastrointestinal (GI) inflammation and bloody colitis. These disruptions to GI barrier function and gut homeostasis coincide with reductions in commensal bacterial diversity. HCT patients with the lowest scores for gut microbiome diversity often have the highest incidence of GVHD-related mortality. Previous research was conducted on an orally administered human lysozyme milk (hLZ) therapeutic sourced from a line of transgenic goats expressing hLZ in their mammary glands. Using a model of porcine malnutrition with enterotoxigenic E. coli challenge, hLZ milk supplementation was

shown to be potentially efficacious in reconstituting some bacterial diversity loss and disruptions of GI barrier homeostasis. A randomized pilot trial was devised to determine the efficacy and safety of oral delivery of hLZ milk in patients undergoing standard allogeneic HCT. This ongoing study aims to enroll a total of 36 eligible patients (aged 12-60), with patients randomized to the treatment group receving hLZ milk supplementation from day -8 to day 30±2 at discharge. Sampling of fecal material, blood, and urine is conducted on specific days, and adverse events are recorded daily. Fecal samples are extracted for DNA and sent for 16S rRNA amplicon sequencing for microbiome analysis. Community profiling is evaluated through measures of alpha diversity, beta diversity, and differential abundance testing, aimed at elucidating if hLZ supplementation amounted to a significant change in microbial diversity across patients throughout the treatment timeline. Patient outcomes and adverse event incidence are also considered in relation to the 16S rRNA community analysis.

K

Global status of gene edited food animals and their agri/food/feed products

Alba V. Ledesma and Alison L. Van Eenennaam, Department of Animal Science, University of California, Davis, USA

A structured literature review was performed to compile a comprehensive listing of gene edited animals for food and agricultural applications. Initially, a collation of English peer-reviewed publications with the term “Gene Editing” in the medical subject heading (Mesh), or gene edit*, or genome edit* or base edit* in the title or abstract; and targeted to pigs or cows or sheep or goats or chicken or tilapia or salmon was performed in PubMed, excluding any applications that were exclusively for biomedical purposes (e.g. xenotransplantation or medical models). This resulted in 581 results from which were excluded those publications where the edits were performed only in cells, or embryos that did not result in a live animals (e.g. not transferred to surrogates), and examples where edited animals did not survive beyond birth. The remaining 103 publications were mostly CRISPR/Cas9 SDN-1 knockouts, particularly myostatin, with China leading the overall publication count. Further searches were performed including diverse aquaculture species, and less common food animals (e.g. rabbits). Moreover, a survey was developed and sent to listservs of animal and livestock geneticists working in the field (e.g., TARC, AnGenMap). To date, several developed or ongoing research applications have been authorized for commerce, or judged to be “non-GMO” hence conventional, in at least one country including knockout tiger pufferfish and red sea bream in Japan; tilapia, cattle, pigs and horses in Argentina; cattle and tilapia in Brazil, and two knockout PRLR-SLICK cattle were given enforcement discretion in USA. One application, the CD163 deletion PRRSV resistant pig lines is formally in the precommercial stage, and there are proof-of-concept applications in multiple food species testing gene targets for traits of commercial interest. These include disease resistance, yield and productivity, single gender offspring; and infertility coupled with germline complementation in multiple species including fish, chickens, cattle, goats, and pigs.

L

Production of precisely edited dairy cattle with light-colored, low heat-absorbing coats

J. Wei[1], B. Brophy[1], S-A. Cole[1], S. Leath[1], B. Oback[1,2,3], J. Boch[4], D.N. Wells[1] & Götz Laible[1,3]
[1]Animal Biotechnology, Ruakura Research Centre, AgResearch, Hamilton, New Zealand.
[2]School of Sciences, University of Waikato, Hamilton, New Zealand.
[3]School of Medical Sciences, University of Auckland, Auckland, New Zealand.
[4]Institute of Plant Genetics, Leibniz Universität Hannover, Hannover, Germany.

Cattle with black coats absorb twice as much solar radiation as cattle with white coats. The enhanced radiative heat gain experienced by black animals is a contributing factor to heat stress which compromises their welfare and milk production. To rapidly adapt dairy cattle to warmer temperatures, we aimed to introduce a naturally occurring mutation in the premelanosome protein gene PMEL by embryo-mediated genome editing. This mutation deletes leucine 18 in the signal peptide and was shown to cause coat color dilution in cattle. Plasmid-encoded TALENs plus a repair template, specifying the p.Leu18del mutation, were microinjected into zygotes. Injected embryos were biopsied and screened by PCR for complete biallelic precision editing. From these verified embryos, eight non-mosaic calves with precision edited genotypes were produced, including calves from high genetic merit parents. They showed a strong coat color dilution phenotype, which was associated with lower thermal energy absorbance. These results demonstrate that embryo-mediated editing can validate putative causative sequence variants to produce low heat-absorbing calves in a single generation.

M

Androgen receptor developmental timeline and expression patterns in fetal pigs.

Jennifer L. Jankovitz and Elizabeth A. Maga
Department of Animal Science, University of California, Davis, USA

Androgen steroid hormones testosterone (T) and dihydrotestosterone (DHT) support fetal development of the male reproductive tract. Literature establishes fetal pigs lacking androgen receptor (AR) mRNA at 17 days post conception (dpc) and positive at 31 dpc. Gonadal development indicates a need for ARs by 26 dpc, however attempts to generate androgen-receptor knock-out (ARKO) swine using CRISPR/Cas9 indicates earlier AR signaling. Established pregnancies failed to survive beyond 30 dpc with guides targeting both AR signaling pathways, suggesting fetal lethality. The current objective is to characterize fetal porcine AR development as it is hypothesized that ARs appear prior to 31dpc. Characterizing timing and quantification of porcine AR expression will help better understand androgenic support in-utero.

Pre-pubertal gilts were artificially inseminated (AI) for time fetal collections. Pregnant reproductive tracts were collected at 16-21 dpc, 24 dpc, 27 dpc, and 29 dpc with day 0 referring to 24 hours after AI. Sub-samples of each litter were flash-frozen for total RNA isolation, cDNA synthesis, and subsequent use in RT-PCR and RT-qPCR for AR expression. Fetal tails were flash frozen for sex determination via PCR with primers for the SRY gene and the Zfx/Zfy gene as a control. Primers for swine AR exon 3 and 4 determined the presence of AR mRNA using RT-PCR, additional primers targeted GAPDH as the house keeping gene. Results were visualized on an agarose gel using electrophoresis. Expression was

quantified using RT-qPCR with primers for AR mRNA exon 6 and 7 and GAPDH. Remaining fetuses were fixed in paraformaldehyde and processed for immunohistochemistry (IHC). Fetal pig AR mRNA has been present in 16 - 21, 24, and 27 dpc fetuses. Preliminary RT-qPCR results indicate a notable increase in AR expression in 24 dpc fetuses when compared to 16 dpc however, full quantification of AR mRNA expression has yet to be completed.

N

Generation of CD209 gene-edited knockout sheep via electroporation

Mitchell L. Angove[1], Thomas F. Bishop[1], Austin R. Brown[1], Oluwafemi A. Falade[1], Bret R. McNabb[2], and Alison L. Van Eenennaam[1]

[1] Department of Animal Science, University of California, Davis CA 95616 USA

[2] Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis CA 95616 USA

Bovine leukemia virus (BLV) is a retrovirus that infects cattle causing significant economic and health concerns for the dairy industry. In the United States, 90% of dairy herds are seropositive for BLV with a 40% intraherd infection rate. The CD209 gene encoding a C-type lectin receptor known as dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) is expressed on the surface of dendritic cells, which are important immune cells involved in the recognition and capture of pathogens. BLV exploits DC-SIGN to attach and enter dendritic cells, facilitating viral dissemination throughout the host. Sheep have been widely used as an important model organism for BLV in cattle. Sheep experience BLV infection similarly to cattle, but show heightened susceptibility and an accelerated disease course. We hypothesized that a CD209 knockout will create disease resistant sheep as the virus will be unable to bind and enter the cell. The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated system protein 9 (Cas9) system and a single guide RNA (sgRNA) approach was used to knockout CD209 in sheep embryos. Three sgRNAs targeting CD209 were selected using software called Chop-Chop and electroporated as Cas-gRNA ribonucleoproteins into sheep zygotes 6-hours post fertilization. Blastocysts were genotyped by PCR amplification followed by Sanger Sequencing. Guide 3 was chosen due to the highest knockout efficiency (~90%), and the lack of homology to potential off-target sites in the reference ovine genome. Embryos were produced using abattoir derived oocytes and a total 55 edited blastocysts were transferred to 11 surrogate ewes using established embryo transfer techniques, resulting in 6 pregnancies and 11 embryos. Twelve blastocysts not transferred showed a knockout efficiency of 83%. Currently, five ewes carrying 8 fetuses are due to lamb in August, 2023. Following birth, genotyping and weaning; knockout and unedited control lambs will be challenged intradermally with BLV+ cow blood.

O

CRISPR-Cas DNA and RNA targeting (DART) for disruption of OCT4 in cattle zygotes.

Jada L. Nix, *Gustavo P. Schettini, Savannah L. Speckhart, Alan D. Ealy, Fernando H. Biase*

School of Animal Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

Deletion of DNA segments by CRISPR-Cas remains inefficient with most cattle zygotes retaining a wildtype allele. Maternal mRNAs add another layer of complexity to understanding gene function, as

mRNAs of the target gene may mask knockout (KO) effects. Here, we present efficient genome editing and transcript degradation in cattle zygotes, named CRISPR-Cas DNA and RNA targeting (DART). We used CRISPR-DART to determine the function of OCT4 in cattle preimplantation embryogenesis. CRISPR-DART is a two-step procedure consisting of an electroporation of ribonucleoproteins (RNP) for DNA editing (CRISPR-Cas9D10A + gRNAs) at 14 hours post-fertilization (hpf), and a second electroporation with two RNPs: CRISPR-Cas9D10A + gRNAs, and CRISPR-Cas13a + gRNA for mRNA degradation at 20 hpf. We determined that CRISPR-Cas13a is effective at knocking down transcripts in cattle zygotes by introduction and knockdown of exogenous fluorescent protein mRNAs. The knockdown groups had reduced fluorescence (0.43-fold compared to control zygotes) with no impact on blastocyst development (34.3% Cas13a+mRNA, 33.3% controls). Next, we determined that CRISPR-DART with non-targeting gRNAs maintains blastocyst development on day 8 (28.2% scramble-gRNAs, 30.7% control). CRISPR-DART targeting OCT4 reduced blastocyst development (7.9% OCT4-KO 30.7% control, $P<0.001$) and impaired inner cell mass (ICM) formation. Targeted sequencing revealed 95.8% (23/24) of OCT4-KO blastocysts had at least one deleted segment on the targeted DNA sequence with many deletions greater than 1500 nucleotides. We estimate that 91% (81/89) of blastocysts had no wild-type sequence of the target region. Immunofluorescence assays confirmed absence of OCT4 protein and an obstruction of NANOG expression in OCT4-KO blastocysts. Transcriptome analysis of OCT4-KO blastocysts revealed dysregulation of 158 protein-coding and one long non-coding RNA genes (FDR<0.1), including downregulation of three pluripotency markers (CACHD1, DPPA4, RRM2). Overall, CRISPR-DART produces biallelic deletion and transcript knockdown in cattle zygotes. Without OCT4, cattle embryos may form a blastocoele but lack a functional pluripotent ICM.

P

A bovine embryonic stem cell reporter line for assessing CRISPR-Cas9 editing efficiency

Dawn Cayabyab, Pablo J. Ross and Elizabeth A. Maga
Department of Animal Science, University of California, Davis, USA

Targeted gene insertion in livestock holds promise for advancing desirable traits and creating biomedical models yet remains a challenge. Introduction of CRISPR-Cas9 genome editing reagents into embryonic stem cells, followed by nuclear transfer cloning, can provide an efficient approach for producing transgenic livestock species that are limited by long generation intervals. To rapidly quantify editing efficiency and expedite optimization of CRISPR-Cas9 technology in cattle cell lines, a fluorescence reporter system in bovine embryonic stem cells (bESCs) was generated by stable integration of the enhanced green fluorescent protein (EGFP) gene driven by the CAG promoter at the Hipp11 (H11) safe harbor locus. Cas9 plasmid, gRNA targeting the H11 locus (H11-gRNA), and an EGFP donor template for homology-mediated end joining were delivered into different bESC lines using lipofection under feeder-free conditions. Transfection efficiency was estimated by fluorescence microscopy 24 hours after lipofection and varied between cell lines, ranging from 15-30%. EGFP-bESCs were isolated from the polyclonal population by limiting dilution and site-specific insertion of CAG-EGFP at the H11 locus will be verified by junction PCR and Sanger sequencing. Preliminary analysis of Cas9 editing efficiency at the H11 locus was done in sorted bESCs lipofected with Cas9-H11-gRNA plasmid only. TIDE analysis of the H11 region showed that Cas9 plasmid produced an average indel rate of 73.8%, confirming activity of Cas9 at the target site. However, to maximize the efficiency of Cas9-mediated knockouts and knock-ins with ease, delivery strategy, Cas9-H11-gRNA format, and DNA donor template will be optimized in monoclonal EGFP-bESC reporter lines.

Q

Gene family ablation by utilizing Cytidine Deaminase CRISPR technology

Benjamin M. Nelson[1], Kevin Wells[1], Ky G. Pohler[2], Randall Prather[1], Bhanu P. Telugu[1], Jonathan A. Green[1]
[1]Division of Animal Sciences, University of Missouri; Columbia, MO
[2]Department of Animal Science, Texas A&M University, College Station, TX

Porcine pregnancy associated glycoproteins (PAGs) are aspartic proteinases abundantly produced by the conceptus and placenta of even toed ungulates. These proteinases represent a gene family that consists of closely related paralogs and members differ by as much as 10 percent. The purpose of this study was to ablate some, or all, of the PAGs in swine to gain insight into their function. To inactivate these gene products, base editing with a Cytidine Deaminase (BE4-Rra3f F130L) was used. Cytidine deaminase works by switching a cytosine to a thymine. These targeted base edits were used to introduce a premature stop codon. The guide RNAs used were designed to target multiple PAG genes. Ultimately, nine of the 13 PAG genes could be targeted with two guides. The first guide was against one of the canonical PAGs (PAG6); this guide was also predicted to target four other PAG6-like genes. The second guide was designed to target another major PAG (PAG3), as well as three other PAG family members. Editing efficiency was evaluated with a PCR screen, followed by sequencing. RNA-seq has been used to confirm knock out efficiency as well. Initial screens showed efficiencies of 72.95 percent to PAG6, while the other PAG6-like targets were not edited, despite that they share 100% identity for the guide target sequence. Endometrium collected from surrogates containing edited embryos contained dilated blood vessels and an expanded subepithelial vasculature; some individuals also contained what appeared to be free blood in the interstitium. Further research will be conducted to explore the mechanism of this phenotype.

R

Use of emerging CRISPR/Cas9 technology to improve homology directed repair to generate transgenic pigs.

Sebastian Lobo and Elizabeth A. Maga
Department of Animal Science, University of California, Davis, USA

Pigs are a major species used as biomedical models and for agricultural purposes. Due to their anatomical and genetic similarity to humans, their translational uses include disease mechanisms, medical procedures, and xenotransplantation. Agriculturally, they are the second largest source of animal protein produced worldwide. Advances in assisted reproductive technologies combined with gene-editing procedures improved the specificity and efficiency of knock-out (KO) animals' generation. However, editing efficiency to generate knock-in (KI) animals through homology-directed repair across all gene editing methods and species remains low.

Standard Cas9 (SD Cas9) is being compared to GeneTether™ (GT Cas9) proprietary technology, designed to increase efficiency of targeted genome editing. This method "tethers" the donor DNA template to the Cas9 protein, positioning tphe DNA template directly at the site of double strand break, by using an E. coli lacI (lactose repressor)-Cas9 fusion protein bound to DNA templates containing E. coli lactose operon operator (lacO) sequence at one end of the dsDNA template. It is hypothesized that zygotes

edited with GT Cas9 would integrate the template with higher competence, suggesting an innovative technology to improve the efficacy of KI in pig embryos.

The lacI-Cas9 fusion protein has been shown, by surface plasmon resonance, to bind lacO DNA sequences comparable to native lacI protein. We demonstrate here that the Cas9 activity is intact and generates KO in embryos similar to SD Cas9. Studies to determine KI efficiences are underway. Oocytes are aspirated from abattoir ovaries and matured in-vitro. After parthenogenic activation, Cas9 RNP and gRNA (2:1), was introduced via electroporation using GT Cas9 and SD Cas9 with a gRNA targeting the androgen receptor gene. Embryos were cultured to blastocyst stage, then lysed using Lucigen QuickExtract and the targeted region amplified via PCR. The presence of edits is determined by Sanger sequencing and analyzed using ICE and TIDE Analysis.

S

Consideration of benchmarks for assessing environmental risks of genetically engineered animals

Sean Ryan, Exponent

As stipulated under the National Environmental Policy Act, the Food and Drug Administration must assess whether the agency action (approval of gene edited and transgenic animals) is likely to have a significant impact on the human environment in the United States. As part of that environmental assessment, and in cases where there is not complete sterility, two common environmental concerns of genetically engineered animals often emerge when considering the possibility of escape into the wild: either through competition or introgression, the genetic alterations and associated phenotype(s) they produce, will either harm desired/beneficial/in-periled wild populations (e.g., endangered salmon) or create new and/or enhanced traits that result in negative impacts to the environment (e.g., wild pigs in North America). Appropriately, risk of potential harm is often evaluated by comparing the risks posed by animals possessing the genetic alteration to a benchmark—animals that do not (status quo). This benchmark is critical for distinguishing between the risk posed by the genetic alteration per se, and the risks posed by current attributes (genomic and phenotypic) of the animal. However, conventional breeding programs have, and continue to, generate an enormous breadth of variation in hundreds of phenotypic traits for any one animal species. Here we consider how this existing variation could be used to provide greater context when developing a benchmark. For example, if the concern is that escaped individuals, or wild populations receiving the genetic alteration via introgression, would result in increased reproductive output, the degree of change in reproductive output should be put into the context of existing variation in traits that influence reproductive output. If this additional context is to be incorporated into the development of appropriate benchmarks in the risk assessment process, it will be necessary to develop a more comprehensive understanding of existing animal genomic and phenotypic variation.

T

Generation and characterization of type I and type III interferon (IFN) knockout chickens

Mohanned Alhussien[1], Romina Klinger[1], Hanna-Kaisa Vikkula[1], Lea Radomsky[1], Iulia Poplacean[1], Hicham Sid[1] and Benjamin Schusser[1]

[1]Reproductive Biotechnology, Department of Molecular Life Sciences, TUM School of Life Sciences Weihenstephan, Technical University Munich, Liesel-Beckmann Strasse 1, 85354 Freising, Germany

Interferons (IFNs) play a critical role in innate immunity against viral infections. Virus-induced IFNs consist of a complex mixture of type I IFN (IFN-α) and type III IFN (IFN-λ). While the receptor for type I IFN is ubiquitously expressed, the receptor for type III IFN functions primarily in epithelium-rich organs, such as the respiratory and gastrointestinal tracts. In contrast to mice models, the role of the chicken IFNs and their receptors are not fully understood due to the lack of investigative tools such as genetically modified chickens. The objective of this project is to generate IFN-α and IFN-λ receptor knockout (KO) chickens using CRISPR/Cas9 technology to dissect the role of avian IFNs in viral infections. Primordial germ cells (PGCs) were co-transfected with a CRISPR/Cas9 vector expressing a single guide RNA and Cas9-2A-eGFP targeting the coding region within exon1 of IFNAR1 chain of the IFN-α receptor and the epithelium-specific chain (IFNLR1) of the IFN-λ receptor. The IFNAR-/- chickens are healthy, develop normal body weight and have normal blood immune cell populations as compared to WT chickens. The IFNLR-/- chickens are currently under close surveillance for further characterization. The IFNs KO chickens will serve as a novel tool to study the avian IFN system and characterize the antiviral properties of IFNs which is crucial for developing potential therapeutics for a wider range of viruses with both veterinary and zoonotic significance.

Participants, TARC XIV 2023

First Name	Last Name	Affiliation	Email Address
Montana	Altman	University of California, Davis	mdaltman@ucdavis.edu
Mitchell	Angove	University of California, Davis	mlangove@ucdavis.edu
Michele	Ardizzone	EFSA	michele.ardizzone@efsa.europa.eu
Benjamin	Beaton	Genus R&D	Benjamin.beaton@genusplc.com
John	Bianchi	Revivicor, Inc.	jbianchi@revivicor.com
Camila	Bonemann Bender	University of Missouri	cbbczbg@missouri.edu
Kelsey	Briggs	U.S. National Poultry Research Center	kelsey.briggs@usda.gov
Diane	Broek	Trans Ova Genetics	diane.broek@transova.com
John	Buchanan	Center For Aquaculture Technologies	jbuchanan@aquatechcenter.com
Shane	Burgess	The University of Arizona	sburgess@cals.arizona.edu
Dan	Carlson	Recombinetics	dan@recombinetics.com
Dawn	Cayabyab	University of California, Davis	ddcayabyab@ucdavis.edu
Arjun	Challagulla	CSIRO	arjun.challagulla@csiro.au
Michela	Ciccarelli	Washington State university	michela.ciccarelli@wsu.edu
A. Mark	Cigan	Genus Research and Development	mark.cigan@genusplc.com
Caitlin	Cooper	Consultant	ccooper@ucdavis.edu
Emily	Cornwell	FDA	Emily.Cornwell@fda.hhs.gov
Jessica	Cruvinel	University of Missouri	jessicacruvinel@missouri.edu
Yifan	Dai	Nanjing Medical University	yifandai08@hotmail.com
Anna	Denicol	University of California, Davis	acdenicol@ucdavis.edu
Dorian	Dincuff	Pharming Group N.V. Branch	d.dincuff@pharming.com
Michael	Ellis	DEFRA	michael.ellis@defra.gov.uk
Kyeong-hyeon	Eom	LARTbio inc.	djarudgus@snu.ac.kr
Dan	Faber	Trans Ova Genetics	dan.faber@transova.com
Oluwafemi	Falade	University of California, Davis	oafalade@ucdavis.edu
Mark	Fife	Aviagen	mfife@aviagen.com
Tatiana	Flisikowska	Technical University of Munich	tatiana.flisikowska@tum.de
Mary	Garry	University of Minnesota	garry002@umn.edu
Gyeongmin	Gim	Lartbio	tty4447@naver.com
Caroline	Gomes Lucas	University of Missouri	lucascg@missouri.edu
Evan	Grusenmeyer	Univeristy of Missouri	grusenmeyered@mail.missouri.edu
Perry	Hackett	University of Minnesota	hacke004@umn.edu
Eric	Hallerman	Virginia Tech University	ehallerm@vt.edu
Jeong Hoon	Han	Penn State University	jqh6364@psu.edu
Jae Yong	Han	Seoul National University	jaehan@snu.ac.kr
Andrew	Hanrtn	The University of Edinburgh	andrew.hanton@ed.ac.uk
Bo	Harstine	Select Sires Inc.	BHARSTINE@SELECTSIRES.COM
Sadie	Hennig	eGenesis	sadie.l.hennig@gmail.com
Goo	Jang	Seoul National University	snujang@snu.ac.kr
Jennifer	Jankovitz	University of California - Davis	jljankovitz@ucdavis.edu
Woldeyesus	Jinore	African Union Development Agency-NEPAD	woldeyesuss@nepad.org
Matt	Josephson	CAT	mjosephson@aquatechcenter.com
Kimon	Kanelakis	FDA	kimon.kanelakis@fda.hhs.gov

Darrell	Kapczynski	USDA	darrell.kapczynski@usda.gov
Keitaro	Kato	Aquaculture Research Institute, Kindai University	kei-kato@kindai.ac.jp
Tae Hyun	Kim	Penn State University	taekim@psu.edu
Cody	Kime	TRANS OVA GENETICS	CODY.KIME@TRANSOVA.COM
Kayla	Knilans	USDA	kayla.j.knilans@usda.gov
Ilektra	Kouranti	Pharming Group N.V. Branch	i.kouranti@pharming.com
Daniel	Kovich	USDA/FAS/TPGA	Daniel.Kovich@usda.gov
Goetz	Laible	AgResearch	goetz.laible@agresearch.co.nz
Alba	Ledesma	University of California, Davis	avledesma@ucdavis.edu
Kiho	Lee	University of Missouri	kiholee@missouri.edu
Hyeyoung	Lee	University of Missouri	leeye@missouri.edu
Phil	Leighton	OmniAb, Inc.	pleighton@omniab.com
Simon	Lillico	The University Of Edinburgh	simon.lillico@roslin.ed.ac.uk
Sebastian	Lobo	University of California, Davis	selobo@ucdavis.edu
Elizabeth	Maga	University of California, Davis	eamaga@ucdavis.edu
Chantal	March	AquaBounty Canada Inc.	cmarch@aquabounty.com
Maciej	Maselko	Macquarie University	maciej.maselko@mq.edu.au
Michael	McGrew	The Roslin Institute and University of Edinburgh	ssisanachandeng@nas.edu
Tomislav	Modric	FDA	tomislav.modric@fda.hhs.gov
Anne	Muia	National Biosafety Authority, Kenya	amuia@biosafetykenya.go.ke
Bill	Muir	Purdue University	ssisanachandeng@nas.edu
Jim	Murray	University of California, Davis	jdmurray@ucdavis.edu
Benjamin	Nelson	University of Missouri	bmnrzq@umsystem.edu
Clint	Nesbitt	Genus PLC	clint.nesbitt@genusplc.com
Jada	Nix	Virginia Tech	jadalnix@vt.edu
Theresa	Pauli	School of Life Sciences, Weihenstephan, Technical University of Munich	theresa.pauli@tum.de
Brent	Pepin	Cytotheryx, INC.	brent.pepin@cytotheryx.com
Carlos	Pinzon	UT Southwestern Medical Center	carlos.pinzonarteaga@utsouthwestern.edu
Irina	Polejaeva	Utah Stae University	irina.polejaeva@usu.edu
Caroline	Povey	Policy/Regulation	Caroline.povey@defra.gov.uk
Ashutosh	Pudasaini	CAT	apudasaini@aquatechcenter.com
Alex	Quinn	Commonwealth Scientific and Industrial Research Organisation (CSIRO)	alex.quinn@csiro.au
Bethany	Redel	USDA-ARS	Bethany.redel@usda.gov
Penny	Riggs	Texas A&M University	ssisanachandeng@nas.edu
Pierre-jean	Ripoll	Pharming	p.ripoll@pharming.com
Diego	Robledo	Roslin Institute, University of Edinburgh	diego.robledo@roslin.ed.ac.uk
Chris	Rogers	Precigen, Exemplar	chris.rogers@exemplargenetics.com
Pablo	Ross	STgenetics	pross2@hotmail.com
Sean	Ryan	Exponent	sryan@exponent.com
Albaraa	Sarsour	National Academies of Sciences, Engineering, and Medicine	Asarsour@nas.edu
Sabrina Teresa	Schleibinger	Technical University of Munich, Reproductive Biotechnology	sabrina.schleibinger@tum.de
Hicham	Sid	Technical University of Munich	Hicham.Sid@tum.de

Samantha	Sisanachandeng	National Academies of Sciences, Engineering, and Medicine	ssisanachandeng@nas.edu
Anna	Spate	University of Missouri, NSRRC	lichtenaueram@missouri.edu
Christine	Tait-Burkard	The University Of Edinburgh	christine.burkard@roslin.ed.ac.uk
Bhanu	Telugu	University of Missouri	telugub@missouri.edu
Mark	Tizard	CSIRO	mark.tizard@csiro.au
Scott	Tyack	Aviagen	styack@aviagen.com
Theresa	Von Heyl	Reproductive Biotechnology, TUM, Munich, Germany	Theresa.vonHeyl@tum.de
Christine	Vuong	OmniAb, Inc.	cvuong@omniab.com
Mark	Walton	AquaBounty	Mmwalton@aquabounty.com
Evelyn	Weaver	Penn State University	euw70@psu.edu
Austin	Weber	University of California Davis	aeweber@ucdavis.edu
Kevin	Wells	University of Missouri, NSRRC	wellskev@missouri.edu
Matthew B.	Wheeler	University of Illinois, Urbana Champaign	mbwheele@illinois.edu
Kristin	Whitworth	University of Missouri, NSRRC	WhitworthK@missouri.edu
Aspen	Workman	USDA, ARS, US Meat Animal Research Center	aspen.workman@usda.gov
Diane	Wray-Cahen	USDA	diane.wray-cahen@usda.gov
Soo-Young	Yum	LARTBIO Co., Ltd.	sy.yum@lartbio.com
Ye	Zhang	Aquabounty	yzhang@aquabounty.com

2023 Transgenic Animal Research Conference

TARC XIV

THANK YOU FOR ATTENDING!

UNIVERSITY OF CALIFORNIA
Agriculture and Natural Resources

UCDAVIS
DEPARTMENT OF ANIMAL SCIENCE