Eunice Kennedy Shriver National Institute of Child Health and Human Development

Physiomimetics and Organoids for Reproductive Health

Virtual Meeting

September 21 and 24, 2021

Day 1

Welcome

Louis DePaolo, Ph.D., Chief, Fertility and Infertility Branch, Eunice Kennedy Shriver *National Institute of Child Health and Human Development (NICHD)*

Dr. DePaolo gave a brief overview of developments in the field of *in vitro* organ systems. Early attempts at creating organoid cultures used large tissue fragments, but these models did not perfuse oxygen well. Cell isolation techniques spurred exciting developments based on examining cell function in primary cell cultures or cell lines. However, cell lines do not adequately represent the multicellular, 3D structure of organs. In the last 5 to 7 years, researchers have developed organs on chips and organ cultures—physiomimetic models—to study biology and function.

The meeting aligns with the NICHD Strategic Plan, specifically the following themes and goals:

Theme

- Understanding the molecular, cellular, and structural basis of development
- Promoting gynecologic, andrologic, and reproductive health

Implementation Goal

- Develop innovative *in vitro* model systems to study gametogenesis and gynecologic disorders

Aspirational Goal

- Accelerate efforts to definitively diagnose, prevent, and treat endometriosis

The goal of the workshop is to bring together basic and clinical investigators working in developing physiomimetic systems that can be used to enhance our understanding of basic endometrial biology, gynecologic pathology, and gametogenesis and to identify a range of key factors involved in these processes and tissues.

Keynote Address

Clinical Mimicry in Human Organ Chips: From Lung to Reproductive Organs

Donald E. Ingber, M.D., Ph.D., Founding Director, Wyss Institute for Biologically Inspired Engineering, Harvard University

Dr. Ingber reviewed the work in his lab building different *in vitro* model organ systems over the last decade. Developing new drugs is costly (more than $3 billion per drug), animal studies are time consuming and result in countless animal lives lost, and up to 95% of the time, the results obtained in animals are not reflected in the clinical response. These factors drive the need for alternative testing models. However, other reasons have emerged. Biologics, for which in many cases there is no appropriate animal model, now make up 40% of drugs in the development pipeline, and there is no good test that accounts for the microbiome, which differs between species, between individuals, and within an individual over time. The microbiome is also particularly important for reproductive health. Chips that accurately model organ function could accelerate drug development, replace animal testing, and advance personalized medicine.

Wyss Institute researchers have been working to engineer such microchips with four broad goals in mind: Recapitulate human physiology and disease states, predict human drug and radiation responses using clinical dose exposures, develop personalized disease models, and create a human testbed to study host–microbiome interactions *in vitro*. The first organ they modeled was the lung, recreating alveolar function on a chip. The chip is made of silicon rubber and consists of three channels, one of which contains a platform lined with lung cells on one side and capillary cells on the other. Breathing is recreated by applying a vacuum in the other two chambers. Air and a medium mimicking blood can also be flowed through the central chamber. Results have demonstrated the human lung's inflammatory response to infection.

The lung model was further developed in collaboration with pharmaceutical companies to model toxicity-induced pulmonary edema, based on their expressed interest in toxicity and disease models. The physiological response in the chip to a typical drug dose was similar and followed the same time course as in a patient. Additional work with GSK on inhibiting the edematous response has led to Phase II trials. This initial model demonstrated proof of principle for disease modeling, drug toxicity, drug efficacy, therapeutic target discovery, new drug discovery, and delivery of gene therapy.

Modeling small airways to study asthma and chronic obstructive pulmonary disease (COPD), researchers were able to grow differentiated cilia with directional movement that cleared mucus at the same rate as in people and to recapitulate exacerbations from tobacco smoke using tissues from COPD patients. Such findings illustrate the possibility of comparing before and after states for an individual with fewer confounders than in a study with patients. Additional studies with samples from cystic fibrosis patients are in review.

Work supported by NIH and the Defense Advanced Research Projects Agency (DARPA) further refined the model for the study of viral infection of the human airway, for research into potential future pandemic viruses. In addition to changes in cell structure, cytokine levels, viral

clearance, and physiological reaction to drug "treatment," drug resistance and viral evolution were accurately modeled. In February 2020, the lab pivoted to studying the effects of drugs with reported effectiveness against other coronaviruses or Ebola virus on a virus with the SARS-CoV-2 spike protein. Testing the screening candidates in a chip and in animal models provided evidence for the effectiveness of amodiaquine, which is currently being studied in clinical trials.

Personalized disease models have been developed using patients' bone marrow to show blood cell development and response to drugs given at clinically relevant doses, demonstrating improvements over gel or suspension cultures. An investigation of an unusual toxicity response that AstraZeneca documented in a Phase I trial of a cancer drug identified a possible mechanism, something that would have been impossible with a suspension culture. Analyzing samples of patients with the rare Shwachman-Diamond syndrome—research conducted in collaboration with Akiko Shimamura and Carl Novina at Boston Children's Hospital and Dana–Farber Cancer Institute—led to the discovery of sub-phenotypes in this small patient population. Researchers in the lab have also developed a human lymphoid follicle chip that enables modeling of the immune response to vaccines.

Organ models for the intestine have been used to culture and study the microbiome—specifically, inflammatory bowel disease, necrotizing enterocolitis, and environmental enteric dysfunction, a condition linked with childhood malnutrition. Researchers have been able to faithfully recreate the intestinal mucous layer; they have also recreated an environment that can sustain both aerobic and anaerobic bacteria, which cannot be done with conventional cell cultures. These chips can be used to identify microbes that mediate physiological effects and metabolites, which could be used to screen candidate therapeutic targets. Multiple chips created from individual samples can also help identify microbiome variations.

In that last couple of years, the lab has developed chips modeling reproductive system biology—specifically the vaginal microbiome—in partnership with the Bill & Melinda Gates Foundation. The aim is to test candidate probiotic therapies for vaginal dysbiosis, a risk factor for infection and preterm birth, in hopes of advancing them to clinical trials. Researchers have also developed a cervix chip.

In total, the lab has developed about 20 organ chips. Research connecting different organs will be used to study organism-level physiology and pharmacokinetics. A robot delivers medium to each chip in turn, which allows for testing at any point and for reordering of the flow. The team aims to use computational modeling and drug levels quantified by mass spectrometry to predict pharmacokinetic parameters.

Given these advances, it is reasonable to argue that these organ chips are ready to be used in drug development and should, in fact, replace animals for validation. Organ chips could even be used to develop drugs for narrowly defined subpopulations. Hospitals are beginning to explore the use of these chips as a personalized medicine tool.

Questions and Answers

Moderator: Candace Tingen, Ph.D., Program Official, Gynecologic Health and Disease Branch, NICHD

Q: Amander Clark, Ph.D. (University of California, Los Angeles), asked how the researchers account for variation among patients who donate tissue or cells for organoid assays.

A: Dr. Ingber said that his team usually gets tissue for organoids from four to six donors and selects the tissue that shows the most robust growth and differentiation.

Q: Regarding the cystic fibrosis experiments, Ann Harris, Ph.D. (Case Western Reserve University), asked whether the cells used were cultured human bronchial epithelial (HBE) cells or cells taken directly from patients. The cell types in HBE cultures vary widely from donor to donor. Given that most cell cultures using samples from cystic fibrosis donors have few basal cells, have the experiments included single-cell RNA sequencing (scRNA-seq) analysis to know what cell types are being seeded into the chamber for each donor?

A: Dr. Ingber explained that the studies, funded by the Cystic Fibrosis Foundation, used cell cultures from patient samples. Although scRNA-seq analysis was not performed, differentiation of multiple cell types was confirmed. Study details can be found online at medRxiv and have been submitted for publication in the *Journal of Cystic Fibrosis*.

Q: Alison Harrill, Ph.D. (NICHD), asked about drug toxicity studies using organ chips. Has the lab characterized *in vitro* drug disposition that might affect drug concentrations both intracellularly and in the media?

A: Dr. Ingber noted that the researchers have done mass spectrometry on the medium in both channels, and his team has done pharmacokinetic modeling. Quantifying amounts in cells could be done easily.

Q: Goli Samimi, Ph.D., M.P.H. (National Cancer Institute [NCI]), asked whether there are molecular or cellular characteristics of organs that influence how well an organ can be recapitulated on a chip.

A: Dr. Ingber said that the researchers have had success with every organ they have attempted to model—and other labs have created still other models. Models do not include every feature of the original organ. Because cells are varied and adaptable, it is not necessary to recreate all features to achieve a functioning organ. The physical environment, flow, and oxygen gradient are essential features and must be examined empirically in order to create a successful model. If a chip does not successfully mimic the original, the next step is to study the problem and determine what to add. Even so, some limitations, including circadian rhythms and whole-body hormonal variations, can be expected to remain at least in the short term.

Q: Dr. Samimi also asked how the cost of organ chips compares with animal studies.

A: Dr. Ingber noted first that studies with chips can be more reproducible than those with animals, because of variance from animal to animal. Commercial chips are expensive, but the

cost can be expected to fall due to manufacturing economies of scale. Ultimately, the cost of chips is likely to fall below the costs of housing and caring for animals.

Q: To understand developmental toxicity, placental transfer of drugs is a central aspect, an attendee commented. With barrier models that contain an artificial force membrane, are there issues with nonspecific drug binding or limited permeability?

A: The chips have 7-micron holes, through which cells can pass, so limited permeability is not a concern. Some drugs can be absorbed by the silicon rubber from which the chips are made. This amount can be measured by mass spectrometry and accounted for in computational models but might pose problems. The postdoc who first developed the lung chip is using the same chip for ongoing work on placental models.

Q: An attendee asked how the medium is selected to support multiple cell types and cell cultures.

A: Designing a chip with two channels and an endothelium layer makes it possible to use a universal blood substitute and a medium that keeps all of the endothelium alive in multi-organ systems. In some cases, the researchers arrive at a viable medium to flow through the epithelial channel by starting with a 1:1 mixture of media for different cell types.

Gametogenesis

Moderator: Ravi N. Ravindranath, D.V.M., Ph.D., Program Director, Preimplantation Genetics and Development and Reproductive Neuroendocrinology, Fertility and Infertility Branch, NICHD

In Vitro Spermatogenesis in Testicular Tissue Organ Culture Systems

Kyle Orwig, Ph.D., Professor, Department of Obstetrics, Gynecology & Reproductive Sciences, Magee-Womens Research Institute, University of Pittsburgh

Dr. Orwig reflected on the trajectory of reproductive technology since the world's first *in vitro* fertilization (IVF) baby, Louise Brown, was born on July 25, 1978. The 2010 Nobel Prize in Physiology or Medicine recognized the research that made her birth possible; since 1978, 6 million babies have been born thanks to IVF.

However, in the United States, 1.3 million men and women between 20 and 50 years old produce either no sperm or no eggs and represent the most difficult types of infertility to treat. The Orwig lab specializes in transplantation techniques allowing patients to use cryopreserved immature testicular tissue to regenerate the production of sperm, ultimately leading to pregnancy. Two technologies now ready for translation to the clinic are spermatogonial stem cell transplantation and autologous testicular tissue grafting. The University of Pittsburgh is now screening patients to identify candidates for these treatments.

For patients with leukemia or cancer of the genital organs and for some transgender patients, these technologies are not appropriate, so methods to mature gonadal tissue or cells so that

they can produce eggs or sperm outside the body are needed. *In vitro* gametogenesis (IVG) from patient-specific stem cells may also be a treatment option for patients who did not preserve gonadal tissues before undergoing a gonadotoxic treatment.

Methods for producing sperm *in vitro* include testicular tissue organ culture and IVG, where primordial germ cell–like cells are derived from patient-derived induced pluripotent stem cells (iPSCs) and differentiated into transplantable stem cells or haploid spermatids or sperm in a dish.

Testicular tissue organ culture was pioneered by Takehiko Ogawa, M.D., Ph.D. In 2011, he reported his success in creating an organ culture system with immature testicular tissue from mice that produced postmeiotic spermatids or sperm. The sperm from these cultures was used to successfully fertilize mouse oocytes and produce normal offspring. Dr. Ogawa then developed a microfluidic device for the culture that allowed him to maintain the tissues for longer. Using sperm collected from tissues that had been maintained for 185 days on the chip, he successfully fertilized mouse oocytes. The pups that were born also matured and produced their own offspring.

Christine Wyns, M.D., advanced this research by culturing immature human testicular tissue on a chip. The tissues were obtained from children undergoing cancer treatment with a potentially toxic effect on their fertility. It was possible to maintain the tissue in culture for several weeks, and differentiated meiotic or postmeiotic cells appeared, but the cultures did not produce sperm. Other labs continue to experiment with techniques for maintaining immature human testicular tissue in culture. Human and mouse adult testicular tissue tends to deteriorate in culture. Importantly, the early successes of generating sperm from cultured tissue and producing live offspring have not been replicated in mice or translated to other species.

IVG from pluripotent stem cells (PSCs) aims to recapitulate normal fetal germline development. Katsuhiko Hayashi, Ph.D., investigated an *in vitro* approach in which he derived embryonic stem cells (ESCs) from a blastocyst-stage embryo. These cells were differentiated into epiblast-like cells, from which primordial germ cell–like cells (PGCLCs) were created. PGCLCs can be further differentiated into spermatids or sperm to create offspring.

Bone morphogenetic protein (BMP) 4 was found to be a critical factor in producing PGCLCs from ESCs. Dr. Hayashi's lab was further able to generate mature spermatids from PGCLCs transplanted into an infertile mouse. These spermatids were used to fertilize oocytes and produce offspring that successfully had offspring of their own. The lab was also able to produce oocytes from PGCLCs.

The laboratory of Qi Zhou, Ph.D., used the same technique to differentiate spermatid-like cells from PGCLCs using fetal somatic cells. Fertilized oocytes produced offspring using this process.

Experiments to produce human PGCLCs have been pioneered by Kehkooi Kee, Ph.D., Dr. Clark, and Renee Reijo Pera, Ph.D., M.S., who demonstrated that BMPs are crucial for the differentiation of germ cells. Azim Surani, Ph.D., CBE, FRS, FMedSCi, helped reveal some of the mechanisms behind the human PGCLC development, including the importance of SOX17 and BLIMP1 in regulating the process.

The research has been translated to nonhuman primates with the production of presumed PGCLCs in a rhesus model. Cells transplanted into mouse seminiferous tubules colonized the tubules and produced typical spermatogonia clusters. In contrast, undifferentiated PSCs transplanted into mouse seminiferous tubules did not. This work was done by the Orwig lab in collaboration with Enrique Sosa, Ph.D., M.S., Gunapala Shetty, Ph.D., Marvin Meistric, Ph.D., Dr. Clark, and others. It is not known whether human PGCLCs can be transplanted or whether they can be differentiated *in vitro* to spermatids and sperm.

Looking ahead, the field will need to address two broad questions: What will the source of human fetal gonadal cells be, and how will researchers test the fertilization potential of human PGCLCs or gametes and the feasibility of producing offspring before they are made available clinically?

A Microphysiological Engineered Approach to Culture Ovarian Organoids

Ariella Shikanov, Ph.D., Associate Professor, Departments of Biomedical Engineering and Macromolecular Science and Engineering, University of Michigan

Dr. Shikanov explained that, with its bioengineering expertise, her lab is focused not on generating new eggs or sperm but on designing extrafollicular or extraspermatozoidal environments. This involves reproductive tissue engineering, or developing and designing new biomaterials and 3D hydrogel-based culture systems.

Fertility preservation for girls and women facing gonadotoxic treatments is the motivation for this work. Many cannot benefit from existing therapies that cryopreserve mature oocytes or fertilized embryos. Two techniques being developed in the Shikanov lab are cryopreservation of a patient's ovarian tissue for later transplantation and isolation of primordial primary ovarian follicles, which can then be cultured *in vitro* to produce mature oocytes that can be fertilized.

Previous attempts to culture mouse follicles in a 2D *in vitro* environment generated flat oocytes and did not translate successfully to higher species. Theresa Woodruff, Ph.D., and Lonnie Shea, Ph.D., introduced the idea of encapsulating follicles in 3D hydrogels, which allowed the oocytes to grow in all directions.

Trying to create a hydrogel that can sustain follicle genesis in three dimensions requires careful consideration of the physical properties of the gel, how to insert and maintain the follicle in the gel, and ultimately how to get the follicle out for fertilization. Early attempts used a plant-based alginate gel. Individual ovarian follicles from mice were placed in alginate, crosslinked, and cultured in wells. Experiments showed that the concentration of alginate, which determines the stiffness of the gel, affects how big the follicles grow. These experiments resulted in the live birth of a mouse in 2006.

These results derived from cultures of late primary and early secondary follicles, but the overwhelming majority of follicles in the ovary are earlier-stage primordial or primary follicles. Culturing individual cells of this size was not successful, but grouping several follicles together in a single hydrogel boosted follicular development. With 10 follicles in a well, all of the follicles survived and grew big enough to ovulate and be assessed for their fertility potential.

Analysis of the secreted cytokines in the media used to culture 5 and 10 follicles showed that grouping 10 together had a synergistic effect on overall development, not simply an additive one. A microarray analysis of transcription factors activated in both cases revealed starkly different signaling patterns for calcineurin, prolactin, and angiogenin. It is conceivable that these factors could be manipulated to improve the *in vitro* growth and development of follicles. Currently, the lab is analyzing the patterns of gene activity to understand the drivers of the different stages of folliculogenesis. This will include RNA sequencing (RNA-seq) analysis of the follicle's somatic cells and oocyte. The ultimate aim of this work is to develop a follicle rescue cocktail to use in the culture.

In considering hydrogels for one or more human follicles, it is crucial to account for the size of a fully developed human follicle—about 2 mm, compared with 350 microns for mice—and develop a material that can expand with the follicle's growth. The lab employed a tissue engineering technique of crosslinking hydrogels with degradable sequences to create a medium that supported mouse follicular growth, polyethylene glycol (PEG) gel. Amino acids can be manipulated to control the degradation. The stiffness of the PEG gel is comparable to alginate, but because of the degradation, it allows for follicular expansion.

To mimic the natural follicular environment, Dr. Shikanov's lab next added small peptides to the culture to retain extracellular matrix (ECM) naturally secreted by the follicle in the PEG gel. This improved maturation rates of the follicles.

About 30 donated human ovary samples are also being analyzed with RNA-seq to identify cell types and markers and to catalog them in a human cell atlas. The samples from the three individuals that have been sequenced so far gave similar results. Most of the cells are stromal cells, and further definition of them is underway. The researchers are also investigating the function of immune cells in the ovary and whether they should be added to culture. The researchers are also exploring where theca cells are coming from.

Biomaterials are also applicable to systems to promote the *in vivo* survival of follicles—for example, for survivors of childhood cancer. Follicles encapsulated in hydrogel are ultimately transplanted.

Ethical Considerations for *In Vitro* Gametogenesis

Insoo Hyun, Ph.D., Professor, Department of Bioethics, School of Medicine, Case Western Reserve University

Dr. Hyun covered the ethical considerations of IVG, which describes the generation of PGCLCs from iPSCs derived from donor fibroblasts. In 2016, the PCGLCs, instead of being transferred into a mouse, were placed in an organoid to derive an egg that was then fertilized.

The organoid method is intriguing from the perspective of human health, because it is likely to have less burdensome regulatory requirements than methods that require transplantation of PGCLCs into the patient's ovaries or testes. However, there are other limitations, such as the Dickey–Wicker Amendment, which prohibits the use of NIH funds for research that creates or destroys human embryos or subjects an embryo to greater risk than it would encounter *in utero*

from the research. This means that federal funds cannot be used for the final stage of testing gametes made by IVG, and this will limit this field going forward.

In addition, the lack of a human embryo research oversight process in the United States is potentially a major limitation to progress in this field. In the United Kingdom, the Human Fertilisation and Embryology Authority oversees embryo research. Institutional review boards (IRBs) are not a good fit, and the stem cell research oversight (SCRO) committees at many institutions may not have the appropriate expertise to review such protocols. There is no obvious solution to this problem, but the easiest approach would be to retool SCRO committees by adding the right expertise. In 2016, the International Society for Stem Cell Research (ISSCR) updated its guidelines for international research for stem cells. The ISSCR broadened the scope of protocol review at institutions to recommend specialized oversight beyond just stem cells to research on mitochondrial transfer and *in vitro* genome editing. The guidelines were updated in 2021 to specifically address IVG.

Funding and oversight must be addressed by the field now, as should the issues of informed consent and sourcing of human cells. Currently, any sample from a patient taken in the course of treatment at a teaching hospital can be used for "education and research," language that is typically included on hospital consent forms for treatment. Legally, this would allow for the use of tissue to derive iPSCs without IRB approval or informed consent, so long as all personal identifying information is disassociated from the sample. However, the Cleveland SCRO committee argued that from an ethical point of view, patients should be able to opt in or out of a process that would create an immortal cell line from their tissue. The committee advised affiliated stem cell core facilities that create IPSCs to get explicit consent from the original donor for iPSC derivation and use. Given that institutions in other cities may still be operating under the conventional policy, which conceivably could cover samples for IVG research, it may be time to revisit this issue.

Other issues may affect the future of IVG research. In the bioethics community, there is growing discussion and little consensus about whether explicit informed consent should be required for IVG research. Some argue that explicit consent is required to derive sperm or eggs from iPSCs. Others would not require it unless researchers proposed to create an embryo (e.g., as a fertilization test). In addition, with the genetic sequencing technology and other resources now available, the time when there are truly anonymous cells may be shrinking. Researchers published an article in *Science* several years ago in which they argued that they were able to find the names and addresses of individual donors from the genetics of their samples and publicly available databases and genealogy sites.

Further in the future, other issues will become increasingly relevant for the field. If generating human gametes becomes possible, it will be important to test whether they can support preimplantation embryo development. Given the prohibition against using federal funds to create an embryo for research, other funders, including possibly the California Institute for Regenerative Medicine, industry, private funders, and even universities, would conceivably support this research from their own funds.

This research can also be expected to be controversial with the public. Some people argue that there is an ethical distinction between creating an embryo specifically for the purpose of

research and using an embryo left over from IVF treatment. In fact, Canada prohibits the creation of embryos solely for research. Years of polling about stem cell research demonstrate that the majority of Americans think that using embryos from a fertility clinic that would otherwise be discarded is acceptable, whereas most think that making embryos specifically for research is unacceptable. This suggests that the environment for IVG research internationally will look very different, depending on where the research is being done, and that the field may face public backlash.

Furthermore, the research community generally follows an agreement to stop growing a human embryo after 14 days in culture or before the primitive streak appears. In some places, including the United Kingdom, this is law. However, as research advances, there may be growing pressure to extend this timeline to include gastrulation, formation of germ layers, and early organ development. It is conceivable that some people will argue that the embryo should be studied for as long as possible before IVG technologies are used clinically. This could apply to developmental biologists as well as researchers interested in modifying embryos using mitochondrial transfer or germline modification with clustered regularly interspaced short palindromic repeats (CRISPR) technology. It is likely that other rationales will arise.

IVG could also potentially be used to generate human gametes in large quantities for other kinds of research. Before the advent of iPSC research, researchers aspired to generate patient-matched cells using the somatic cell nuclear transfer methods that led to Dolly the sheep. This method would have generated stem cells that are a genetic match with the donor. However, limited access to unfertilized human eggs created a bottleneck, and this line of research did not advance. If it becomes possible to generate large numbers of high-quality eggs from IVG, the dynamic could change. IVG research could have the effect of promoting *in vitro* germline modification research—whether for basic research or a preclinical proof of concept, mitochondrial transfer research, or other research that requires a source of unfertilized human eggs.

The field should also explore questions about changes in a gamete's genome due to IVG. Given that spontaneous changes happen naturally in the process of gametogenesis, how many and what types of changes should be tolerated in IVG systems? This will be especially relevant for clinical applications.

Germline editing research for human reproductive purposes may also emerge. Perhaps the safest approach is to alter iPSC lines with CRISPR, analyze the master cell line to make sure all of the changes that occurred are intended, and then derive sperm or eggs from the master cell line. It would be better for the editing to occur early in the research process rather than at fertilization or an early embryonic stage.

It is also important to consider social and cultural impacts of IVG research. Access to these technologies is a pressing concern. What if somatic cell gene editing costs $1 million per attempt? It is also important to keep in mind the concern that assisted reproductive technologies reinforce gender norms and leverage societal pressure to have children or "give" one's parents grandchildren. The extent to which these technologies reinforce gender norms could become an issue and should be considered carefully. At the same time, these technologies make possible parenting arrangements that defy gender norms and expectations,

such as allowing two men or women past the age of fertility to have their own biological children. IVF offers the possibility that many people will play a role in a child's conception and nurturing: The egg donor, sperm donor, surrogate, and parents who raise the child could all be different people. Even postmortem reproduction could be possible. IVG could reanimate debate on these topics. In addition, beyond human reproduction, IVG could conceivably be used for animal species rescue and conservation.

The ISSCR updated its Guidelines for Stem Cell Research and Clinical Translation this year. Of note, proposed IVG research must go through specialized scientific and ethical review if it entails fertilization tests and embryo creation. Other IVG research should be reported to the institution but does not have to be reviewed. Transfer of a human-derived embryo into a human or nonhuman uterus is not permitted.

Gametogenesis Speaker Q&A

Moderator: Ravi N. Ravindranath, D.V.M., Ph.D., Program Director, Preimplantation Genetics and Development and Reproductive Neuroendocrinology, Fertility and Infertility Branch, NICHD

Q: Dr. Clark asked what is missing from testes models that prevents them from producing sperm.

A: Dr. Orwig said it would be logical to test two factors: luteinizing hormone (LH) or human chorionic gonadotropin (HCG) and follicle-stimulating hormone (FSH). The tissue used for the culture system is immature testes, in which Sertoli cells are not mature and Leydig cells do not produce testosterone. Presumably, FSH would promote proliferation and maturation of Sertoli cells, and LH or HCG would stimulate testosterone production, which is essential for spermatogenesis. The testosterone level in testicles is much higher than in the general circulation, so stimulating testosterone production from Leydig cells could be the most effective way forward. Although they have been studied in cocktails of 10 or more factors, these hormones' impact on the tissue has not been studied independently. That will be an important next step.

Q: Dr. Clark asked whether theca cells are still present in the *in vitro* follicles.

A: Dr. Shikanov said that they are. When the secondary follicles are mechanically isolated, theca cells can be observed. It appears that they contribute to the follicular environment: Primary follicles with theca cells rescue follicles that have none. RNA-seq analysis may make it possible to measure the number of theca cells.

Q: Given previous findings showing that an intact follicle was required for the positive growth effect seen with multiple follicles in culture, Francesca Duncan, Ph.D. (Northwestern University), asked whether the transcriptomic analysis from Dr. Shikanov's lab indicated what mechanisms are in play in the crosstalk between granulosa cells and the oocyte.

A: Dr. Shikanov explained that Dr. Duncan's paper showed that if an oocyte alone or the somatic compartment alone is in culture with follicles, the follicles are not rescued. The whole

follicle, with all of the factors it secretes, is necessary to rescue other follicles in coculture. The Shikanov lab's RNA-seq work has just been completed. The oocyte and somatic cells were sequenced separately, which will allow for a comparison of the oocyte, somatic cell, and whole follicle transcriptomes. These results are expected to be published in 2022.

Q: Dr. Harris asked whether Dr. Shikanov and the researchers in her lab have thought about combining the RNA-seq results from follicles with data from other tissues.

A: Dr. Shikanov said that that will be a necessary future step, especially to tease out what is in the soma. Many recent papers have been deciphering the single-cell RNA of different tissues. Theca precursors are not found in other tissues, but immune cells, endothelial cells, and cells related to the lymphatic system or capillaries are similar in other tissues.

Q: Dr. Duncan asked Dr. Shikanov how she envisions integrating, engineering, or modeling the dynamic changes that occur in the follicular and extrafollicular compartments with early development, the menstrual cycle, and aging.

A: Dr. Shikanov said that this exactly describes what the lab is considering doing next. Designing synthetic materials with cell-driven motives is part of this aim. The point of using ECM-binding peptides was to allow cells to secrete ECM that they make and then degrade it. The ECM that follicles secrete, the molecules they deposit, and the growth factors they sequester will be different at different stages of folliculogenesis. It will also be interesting to explore combining different components of the female reproductive tract to model the menstrual cycle.

Q: Dr. Clark asked how historical cell lines (i.e., fibroblasts from banks) and human ESCs (hESCs) would be handled if a recommendation for IVG to require explicit consent were made. Would donors need to be contacted again and reconsented?

A: Dr. Hyun said that the answers for hESC lines and for iPSC lines would be slightly different. The argument for requiring explicit reconsent of the gamete donors for hESC and the couple for whom the embryo was made is weaker, because the source of the cell line—the embryo—has already been destroyed. There is a stronger case for requiring reconsent with iPSC lines, because there is a 100% genetic match. To be consistent, someone who would argue that explicit consent is necessary for IVG research—whether an embryo is created or not—would likely say that explicit reconsent is needed for deriving gametes from iPSC lines. It could also be argued that derivation of the gametes does not require reconsent, but creation of an embryo does. The issue of consent could haunt a researcher's work if they do not get explicit consent in advance. For researchers who plan to do research that involves fertilization, it is hopefully not too onerous going forward to prospectively ask donors for their consent. For example, journal editors or reviewers could raise the issue when findings are submitted for publication. Generally speaking, research that can be done with hESCs can be done equally well with iPSCs. But the ultimate goal of providing fertility treatments is a clear practical reason to prioritize the use of iPSC-derived gametes in research. The bigger issue is whether iPSC-derived cells will be

good enough for clinical use. Jeremy Sugarman, M.D. (Johns Hopkins Berman Institute of Bioethics), added that his research shows that there are common opinions among donors regarding donating tissue to derive iPSCs and for organoid research. In both settings, the results show that donors are generally enthusiastic about the research but have concerns about brain and gamete research. These concerns can be allayed by factors such as ensuring that there is appropriate consent.

Q: Joanna E. Burdette, Ph.D. (University of Illinois at Chicago), asked whether there are major species differences between human and murine ovaries that are being modeled in the ECM for follicle culture.

A: Dr. Shikanov suggested that this is more relevant for cells than for ECM. The distribution of follicles in human ovaries is significantly larger. The medulla contains more stroma, ECM, and large blood vessels. It is as though the human ovary has more of everything compared with the mouse ovary.

Dr. Hyun commented that the 2016 ISSCR guidelines said there may be some exceptions to consent requirements if an important research question can be explored only by using banked, historically significant tissue and if the donor is difficult to reach. These questions are considered on a case-by-case basis. Generally, getting explicit consent is recommended, but if there is enormous scientific value to be gained and no alternative tissue source, and especially if the donor is no longer living, proceeding without consent could be considered.

Listening Session

Moderators: Ravi N. Ravindranath, D.V.M., Ph.D., Program Director, Preimplantation Genetics and Development and Reproductive Neuroendocrinology, Fertility and Infertility Branch, NICHD; and Travis Kent, Ph.D., Program Official, Contraception Research Branch, NICHD

All attendees were asked to comment on where the field of IVG is currently and where it should go. Dr. Shikanov said that 10 years ago, the knowledge of biology was insufficient, so no one was able to reproduce organs like they can today. However, even with multi-omic analyses, the process takes a long time. The computations and expertise required to analyze omics, secreted factors, genetics, and other elements represent a bottleneck. With access to mice and human tissue, the field is now moving faster, but not fast enough.

Dr. Orwig added that despite decades of research on IVG methods, few studies have been replicated. Translating a finding from lower species to higher ones is hard enough, but given that the ultimate goal is to give birth to babies, it will be difficult to argue that the field has gained much of value from the research if achieving that endpoint is not feasible. Being able to test cell omics will not answer the question of whether producing healthy offspring is feasible and safe. While discussing the ethics of this research is important, it is not clear why producing germ cells *in vitro* should be regulated differently than producing any other type of cell. Asking

the donor for permission to produce germ cells is a good idea. But if the egg will not be fertilized, why should there be restrictions on creating germ cells?

Dr. Hyun noted that people could have cultural or religious objections to deriving sperm or eggs, because some people think of these cells differently than they think of other cells. Derivation will have significance for familial or other reasons for some people; out of respect for such individuals, researchers should check with donors.

Dr. Clark added a comment on explicit consent from a researcher's perspective. Consent is also an important part of public trust in research and in the people who conduct it. As Dr. Sugarman noted, the literature documents particular concerns from patients regarding brain organoids and gametes. Researchers working in these areas show their respect for donors by using stem cells in the way that was intended, specifically the way or ways that donors consented to.

In addition to creating germ cells for reproduction, Dr. Shikanov commented that restoration of endocrine function is another important goal of IVG. Hormone replacement therapy has limitations, especially for girls who have undergone puberty or boys who have lost their testes. An *in vitro* follicle would offer reciprocity and dynamic delivery of various hormones at different stages, unlike therapeutic delivery of individual hormones, and could eliminate the need for ovary donors.

Dr. Ravindranath asked Dr. Hyun for more details about potential embryo research oversight bodies at U.S. research institutions, given that law prohibits the use of federal funds for this purpose. Dr. Hyun explained that an oversight system that looks at embryo research protocols would not review NIH-funded research as things stand now but could review research with other sponsors that aimed to create and study human embryos, with the understanding that the embryo would be destroyed in the process. Typically, proposals for research like this come from fertility clinics or local hospitals that want to do research on embryos remaining after the fertility treatment process.

Da-Yu Wu, Ph.D., (National Institute on Drug Abuse), asked how well tissue chip technology has been applied to the study of human placenta. Arum Han, Ph.D., an electrical and computer engineering professor (Texas A&M University), noted that there are a few different systems. One is a vertical coculture system; another is a horizontal system using a microfluidic channel array supporting two or three cell types. However, placenta-on-a-chip systems overall are at an earlier stage of development than other organ chips.

Dr. Duncan commented that early ovary models were simple, but in the last decade, complexity has been engineered back into model systems. The huge quantity of stromal cells shown in the RNA-seq analysis of the human ovary is striking and leads to questions about the diversity of cell types. What efforts are underway to culture primary ovarian stromal cells and preserve their heterogeneity over time? What is the state of research on preserving cell types? Dr. Shikanov explained that earlier work with smaller follicles cocultured with

macrophages showed that the macrophages lose their identity over time and become fibroblasts. Since then, smarter biomaterials that can promote cell binding and ECM deposition have been developed. Models are moving toward a modular approach—from bulk hydrogels to microgels. Microgels are tiny beads (10 to 15 microns in diameter) that can anneal around follicles. The resulting macropore structure allows for better diffusion, and it is possible to encapsulate more follicles and mix and match different particles. These new materials also allow for the formation of layers and concentric centers. Biomaterials have developed a lot since the last time researchers tried to coculture multiple cell types with follicles.

Attendees were asked how reproductive models can be scaled up for use in toxicology and pharmacology studies in infertility and contraceptive research. Dr. Orwig acknowledged the work of Ina Dobrinsky, D.V.M., Ph.D. (University of Calgary Cumming School of Medicine), who showed that testicle cells in suspension can form seminiferous tubules and, in some cases, achieve spermatogenesis. She recently extended these results to *ex vivo* organoids developed from mice, monkeys, and humans. The tubules form inside out—with the basement membrane, Sertoli cells, and embedded germ cells—and can be maintained in multiwell plates. This format could be used to study pharmaceuticals or environmental exposures. Complete spermatogenesis has not been achieved with these models, but they could be used to study effects on cell health.

Dr. Shikanov added that because human reproductive tissues are so much more complex than, lung tissues, for example, it is more difficult to work with the former. Compared with a monolayer of lung cells exposed to medium on one side and air on the other, reproductive tissue models must incorporate cyclical response to hormones and many parts. It is not possible to reproduce the female reproductive system by using microfluidics. The questions to ask with such models need to be simplified in order to get answers. The 3D follicle culture system, for example, is perfect for studying the effect of toxicants and drugs. These models would provide a more direct answer at less cost than a mouse study. However, 3D culture toxicology does miss some elements, such as liver metabolism.

Ji-Yong Julie Kim, Ph.D. (Northwestern University), responded that her lab has developed a female-reproductive-tract-in-a-dish system and is exploring the effects of environmental toxicants. This system uses mouse ovaries that are treated to mimic the endocrine behavior of human ovaries and can look at such questions systematically.

Dr. Duncan added that the power of a follicle culture system is that it can be used independently of the ovary or the rest of the body to study folliculogenesis, oogenesis, ovulation, wound healing, and other processes in a controlled fashion. The assay also provides quantitative metrics, making it possible to track follicle growth and survival, hormone production, meiosis, ovulation, and more. With this system, researchers can get gamete cell, somatic cell, and endocrine readouts, which makes it possible to look at the effects of toxicants, fertoprotective agents, and chemotherapeutics. Because ovulation can be induced in the dish, the system can also be used to screen for agents or pathways that block that process. These simpler assays, along with models of the entire reproductive tract, can be used to probe different areas of reproductive science.

Attendees were asked to discuss the potential of organoid systems for high-throughput screening. What are the barriers to making current technologies scalable for high-throughput therapeutics? Dr. Han commented that in collaborating with life science colleagues to develop tissue chips, a processing capacity of 48 samples is reasonable and efficient from both an engineering perspective and a tissue processing perspective. In the field, many chips have been made into pumpless chip systems, which are complex systems with 48 and in some cases up to 96 wells. Using tissue chips based on a two-tier structure, where simple tissue chip systems are used for primary screening and a more complex chip is used for second-level mechanistic results, may be a good strategy. The field is moving in this direction.

Stephen Palmer, Ph.D. (Baylor College of Medicine), added that high-throughput screening is typically used when there is a specific target in mind. Advanced technologies can be used for much lower throughput—on the order of 50 to 100 compounds, not hundreds of thousands. The exception would be phenotypic screening, when the target is unknown. In that case, researchers could start with representative targets for the top 100 pathways to help narrow the focus. Dr. Palmer's lab used such an approach for a phenotypic screen of rat cells; now they use a lower-throughput evaluation assay of 100 compounds in rat granulosa cells. The scale does not have to be massive to apply to drug discovery.

Dr. Dobrinsky added that micro-organoids on organoid platforms lend themselves to large numbers, but the problem for screening is how to evaluate the endpoint. This is a concern once researchers go beyond mouse studies. Researchers must define the research question carefully.

Vasantha Padmanabhan, Ph.D. (University of Michigan), raised the issue of epigenetics and epigenetic effects on the health of offspring. A healthy baby is not the only relevant outcome; other programming issues deserve attention. Dr. Orwig noted that the studies in his presentation all evaluated genetics and epigenetics to some degree.

In the chat, Dr. Harrill noted a caveat that screening for chemical toxicity in the environmental chemical or contaminant regulatory space may be on the order of hundreds to thousands of molecules. In that case, a tiered screening approach would be preferred. The initial screen would be in a simpler model, and the subset of potential actives would be moved to a higher-order model such as a chip.

Dr. Orwig added that it is difficult to imagine developing a complex chip system for reproductive tissues like the ones Dr. Ingber described in the keynote. Some aspects of germ lineage development can be recreated from cells, but it is extremely difficult to recreate entire tissues for large-scale testing. The question for high-throughput screening then becomes whether there are adequate surrogates for screening that contain a few cell types, such as granulosa cell cultures. Is the objective to recreate an ovary or testis that could be assayed, or would a few relevant cell types assayed at same time, possibly at higher throughput, be adequate for these screens?

Attendees were asked to comment on appropriate endpoints and to discuss how models replicate the biology of the testes. How do researchers decide what outputs to look at? How do newer models compare with traditional *in vitro* models? Dr. Orwig said that for him, the relevant outputs are always reproductive: fertilization and production of live babies. From the perspective of patients with intractable infertility problems, this is what is most important.

Attendees were asked if other endpoints should be looked at in order to eventually make these approaches clinically feasible. Dr. Orwig said that production of gametes is an important output. But even if it cannot be supported by NIH, studying fertilization and early embryo development will be crucial. There are other ways to fund and ethically perform this type of research. It is important that researchers be transparent about their methods and goals to both the community and the organizations in which they work and to take concerns into account when designing experiments.

Dr. Dobrinsky added that even where success has been achieved in mice, further advances have been limited. There is a need for other animal models that can serve as a step between mice and humans, to allow experiments that include fertilization and testing of offspring.

Dr. Hyun asked about the term *primordial germ cell–like cells*. How does its use differ from the term *primordial germ cells*? Additionally, is the aim of research to develop a model that is as close to the real gamete as possible, or is a functional equivalent a reasonable goal? Dr. Orwig said the terminology has become convention in the field. The "-like" terminology acknowledges there is not complete certainty that the *in vitro* and *in vivo* products are the same. The objective is absolutely to produce something exactly like the *in vivo*–derived counterpart. That is not trivial when talking about PGCLCs. It is necessary to study the human fetus to understand human germ lineage development, but getting access to fetuses is difficult and, in some places, impossible. Without a roadmap created by looking at *in vivo*–developed germ cells, it will be impossible to tell whether *in vitro* versions are exactly analogous.

Dr. Clark added of all the iPSC-differentiated cell types, germ line cells are the only ones where *in vitro* versions are referred to with this "-like" terminology. Editors have also emphasized the importance of explicitly distinguishing endogenous from *in vitro* cell types so as not to confuse reviewers and readers. The distinction has been helpful when identifying what is the same and what is different when comparing the two types of cells; over the years, *in vitro* versions have grown closer and closer to the *in vivo* ones.

From the chat, Bo Yu, M.D. (Stanford University Medical Center), asked which platform is more promising for incorporating multiple cell types. The first presentation would seem to suggest that an organ-on-a-chip is better than organoids. Dr. Ravindranath further asked whether a standard organoid or organ-on-a-chip model system has been established or is being created for the ovary or testes. Dr. Orwig said that Dr. Ogawa's work shows that the testes can be recapitulated as an organ-on-a-chip. Immature testicular tissue can be maintained

for a long time in a microfluidic system. The extent to which this system could be scaled up is limited only by access to a source of immature tissue. That is not trivial even for mouse tissue, but it is even more difficult for humans. High throughput would not be feasible for humans for this reason.

Dr. Clark added that the two approaches are complementary techniques. For developmental biology, models are critical, because the organ does not exist anymore. Organoids tend to recapitulate epithelial tissues well, so many are epithelial; organs-on-chips are not restricted in the same way. The relative advantages of each also depend on the research question.

Dr. Dobrinsky added that testes organoids have been made exclusively from primary cells, but other organoids that have supported big research breakthroughs have been made from pluripotent cells differentiated into different cell types. In addition to generating germ cells, different somatic cell types in the testes could be generated, too, and entire organoids could be grown from pluripotent cells. Chip technology could be used for long-term cultures, which could get the field past the hurdle of relying on immature tissue. This is a way forward.

Attendees were asked to comment on where reproductive models have succeeded in incorporating complex niche cells and architectures, and where they can be used to further understand complex niches. Dr. Orwig noted that Dr. Hayashi recently published a paper describing the production of both PGCLCs and pluripotent cell–derived granulosa cells. As Dr. Dobrinsky suggested, it would be possible to produce similar cell types from pluripotent cells in the testes. The two cell types have been created for testes independently, but they have not been brought together into a functional unit. That work still needs to be done.

Attendees were asked what opportunities for organoids or physiomimetics can be most readily realized. Dr. Shikanov responded that folliculogenesis is feasible. Whether it is better to use organoids or microfluidic chips depends on the biological question to be answered. For example, a microfluidic system cannot be used to grow a 5-mm human follicle, because the desired product is too big. It may be necessary to use both microfluidic systems and macro hydrogels, depending on what question is driving the research. The progress in understanding folliculogenesis is breathtaking. Folliculogenesis and the design of new materials are where there has been major progress. It is now possible to do so much more than before. Hopefully, it will be possible to grow fertilizable eggs in synthetic systems and use steroid-producing cells to restore endocrine function. Microfluidics would be well suited to this latter application.

Dr. Orwig said that the important opportunity to grasp—for understanding developmental biology and for clinical application—is to translate organ culture system to higher primates and humans. The potential for this type of system is huge, and current understanding of the system is the only obstacle. If a functional gamete can be produced, working with a system such as nonhuman primates offers the opportunity to fertilize and produce offspring.

Kevin G. Osteen, Ph.D. (Vanderbilt University Medical Center), commented that the human reproductive system essentially sits in idle for 12 to 14 years, which is a long time for infectious

disease or environmental exposure to eventually affect reproduction. One readily accessible area of research is to look at stressors that occur early in life and trace their effect on reproduction years or decades later.

Dr. Hyun underscored the importance of engineering to move the research forward and the need for collaboration between engineers and biologists. He asked whether the remaining problems are mostly engineering issues or questions of biology. How is collaboration fostered? Dr. Orwig commented that because the systems are so complex, it is not necessarily reasonable to think a single lab or even an institution would have all the necessary expertise. The way to move the field forward is to reach across disciplines and make advances faster than working alone. Collaboration also has personal as well as scientific rewards.

Dr. Han described what biologists and engineers offer each other and the close contact that goes on as part of collaboration when developing organoids and chips. Visiting each other's labs to see details in person is helpful. Collaboration works best when the labs are in close proximity.

Dr. Ravindranath asked about the advantages of focusing on culturing follicles, rather than the whole ovary. Dr. Shikanov noted that in mice, secondary follicles, which already have an oocyte, granulosa cells, and theca cells, can grow by themselves in culture. But there are few secondary follicles in the ovary—10 to 15, compared with hundreds of thousands of primordial follicles. Primordial follicles cannot grow on their own, and they rely on other cells that are still being identified.

Day 2

Uterine Biology and Pathophysiology

Physiomimetics at NIH: A Platform for Partnerships

Danilo A. Tagle, Ph.D., M.S., Associate Director for Special Initiatives, National Center for Advancing Translational Sciences (NCATS)

Dr. Tagle presented an overview of the NIH Microphysiological Systems Program, the partnerships that made it possible, the program's development, and its goals for the future. He noted that the mission of NCATS, the newest of NIH's 27 institutes and centers (ICs), is to "catalyze the generation of innovative methods and technologies that will enhance the development, testing, and implementation of diagnostics and therapeutics across many human diseases and conditions." The current tools for drug development using 2D cell culture and animal models are poor predictors of human response.

The NCATS Tissue Chips for Drug Screening Program aims to "develop an *in vitro* platform (tissue chips or microphysiological systems [MPS]) that emulates organ physiology and function using human cells and tissues through advances in stem cell biology, microfluidics, and bioengineering in order to accurately evaluate the efficacy, safety, and toxicity of promising therapies." This can be accomplished by taking an organ system to its basic functional unit and simulating the biomechanics. Multi-organ models can be linked through microfluidic channels.

Interest in the tissue chip program began at NIH even before the formation of NCATS. The investment focus was initially on models for toxicity and later for efficacy and disease modeling. One of the first challenges was monitoring accelerated aging, prompting NCATS to partner with the Center for the Advancement of Science in Space (CASIS) and the National Aeronautics and Space Administration (NASA) to study tissue chips in space. Most recently, funds were obtained from NIH to model addiction systems and Alzheimer's disease. NCATS also funded Tissue Chip Testing Centers and a database center to ensure that the programs developed are fully validated; these programs are now self-sustaining beyond NCATS support.

In addition to partnerships with other NIH ICs, partnerships were formed with the U.S. Food and Drug Administration (FDA) to provide guidance for regulatory approval. In 2014, an IQ Consortium was established with about 21 pharmaceutical companies that expressed an interest in MPS and how they can be used in drug development. Current gaps in pharmacology safety research include assessing toxicity when no pharmacologically relevant models are available; identifying rare or idiosyncratic toxicity of investigational drugs; identifying cardiovascular, hepatic, neuronal, renal, gastrointestinal, and immune toxicities; understanding the human relevance of toxicity in animal studies; and representing disease and population heterogeneity in disease models.

NCATS funding for MPS over its first 5 years focused on drug safety studies. This research showed that it was possible to represent organ function in chips and capture human responses that were not necessarily captured in *in vivo* animal studies. The research then pivoted toward studies of efficacy and disease modeling. Currently funded research (2018–2022) for efficacy

testing and disease modelling addresses many areas, ranging from very rare to very common conditions.

In 2020, in partnership with NCI, NICHD, and the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), NCATS launched Clinical Trials on a Chip to inform clinical trial design and implementation in precision medicine. The goal is to determine whether chips can be used to establish recruitment criteria, stratify patients, and develop clinically relevant biomarkers. The first phase of the award will focus on developing and validating rare pediatric and common disease models containing patient-derived cells. The second phase will involve testing potential drugs for efficacy and safety in clinical trials. The currently funded Clinical Trials on a Chip projects (2020–2025) cover a wide range of metabolic diseases.

The modeling of age-related disorders with NASA and CASIS involved the study of the physiological changes undergone by astronauts under prolonged microgravity. This work provided an opportunity to identify molecular signatures of aging or accelerated aging under microgravity when the chips were returned to Earth. Goals for the platform included ease of use and cost efficiency. NCATS worked with space engineers and other partners to reduce the materials to the required dimensions and specifications. The automation and miniaturization for spaceflight led to greater commercialization opportunities of tissue chip technology and allowed broader adoption and use for tissue chips on Earth. NCATS funded nine age-related projects that addressed immunosenescence, post-traumatic osteoarthritis, drugs that cross the blood–brain barrier, kidney stone formation, lung infection, cardiovascular disease, sarcopenia, and gut inflammation. The data are being analyzed, and publications are forthcoming. Tissue chip models have also been used to respond to national health emergencies, such as the opioid crisis and the COVID-19 pandemic. NCATS formed a new working group of international scientists for MPS COVID-19 research.

The chip platform still needs to be validated so that it can be used for regulatory approval. There must be a clear rationale for the assay, a relationship with endpoints relative to the *in vivo* effects of interest, a detailed protocol, examination of intra- and inter-test variability, assessment of performance against representative compounds, an evaluation according to standards, and the collection of data in accordance with good laboratory practice (GLP). The tissue chip validation framework includes physiological, analytical, and industrial elements.

The Microphysiology Systems Database (MPS-Db) is an integrated database, analytics, and modeling platform. Current MPS-Db content includes a number of datasets, including some available to the general public. Many NIH ICs now have their own MPS programs. The Center for Scientific Review created a dedicated study section that will review applications for MPS. NCATS is also supporting the MPS Global Summit in June 2022 in New Orleans, working with other countries, and continuing to partner with a variety of agencies.

NIH has supported many spinoff and startup companies centered on tissue and organ-on-a-chip technologies. The democratization of the technology platforms allows pharmaceutical companies and other end users to choose from at least 20 companies for services and/or purchase of various platforms and accompanying consumables. The FDA is becoming more aware of the usefulness of this technology; has established an Alternative Methods Working Group, as well as MPS laboratories; and has funded MPS awards for specific applications. The

IQ MPS Affiliate co-authored a series of eight publications on guidelines for using MPS and has planned future articles.

Pharmaceutical companies are using the MPS platform for target identification, lead optimization, preclinical safety and efficacy, and pharmacokinetics/toxicokinetics. Tissue chips may have their greatest impact on drug development, saving an estimated 10% to 26% in costs.

Physiomimetic Models of Endometriosis

Linda G. Griffith, Ph.D., School of Engineering Professor of Teaching Innovation, Biological Engineering, and Mechanical Engineering; and Director, Center for Gynepathology Research, Massachusetts Institute of Technology

Dr. Griffith began her research with physiomimetic models by working with pharma in the 1990s to build a liver model, which has endured and been commercialized around the world. The platform, a 3D liver culture in a perfused format, captured enough of the liver features for studying metabolism, toxicity, and some aspects of immunology. The key invention was a powerful microfluidic pump. The format was extended to a multi-organ format, connecting the liver to the gut and other tissues, which were mostly cells cultured in a Transwell format. This allowed for investigation of multi-organ interactions, such as short-chain fatty acid effects on gut–liver–brain interactions.

Endometriosis-on-a-chip

Dr. Griffith's main focus in getting involved with physiomimetic models was to study endometriosis, a chronic inflammatory disease that affects hundreds of millions of women worldwide with few drugs to treat it. Although endometriosis has been staged according to lesion burden, there is enormous heterogeneity in patient characteristics and clinical symptoms. A better system, much like that used for breast cancer, is needed for classifying endometriosis. Dr. Griffith's research team hypothesized that by looking across networks that involve the immune system and invasion, different groups of patients might be distinguished by different mechanisms. The Systems Biology of Endometriosis project was launched, and patients were classified according to immune signatures in the peritoneal fluid. About one-third of patients in various disease stages were found to have a signature set of cytokines. Macrophages regulated by c-Jun N-terminal kinase (JNK) were producing inflammatory cytokines, suggesting that classification may be possible.

Preclinical trials by Palmer et al. and Hussein et al. showed that JNK inhibitors cured endometriosis in rodents and baboons. These findings encouraged the researchers to build models of the human endometrium, both eutopic and ectopic, to determine whether JNK inhibitors would have clinical uses. A model of the lesion parameter space was built based on biophysical and biochemical cues that defined the lesion environment and could be mimicked *in vitro* in a systematic way. Biopsies of patients' tissues were taken and then reconstructed with tissue engineering so that they could be studied for weeks at a time. The biomaterials included were useful and are in the process of being commercialized. The approach was developed by Jeffrey A. Hubbell, Ph.D., and includes PEG polymer-peptide macromers, a peptide crosslinker, the cells, and light or a pH change. The cell line is encapsulated in hydrogel. A matrix gel was replaced with hydrogel, which is synthetic, reproducible, and easy to use.

Based on the research of Brown et al., a "parameter space" was developed to tailor the matrix for specific applications, while considering cellular (e.g., protease activity), physical (e.g., cross-linked density), and biomolecular (e.g., cross-linked degradability) factors. Matrix-binding peptides were added to the matrix so that when a cell produces fibronectin, the matrix captures and helps sequester it. The synthetic "one-size-fits-all" matrix helps the cell modify the environment to be exactly what it needs. This 3D structure can keep both stromal and epithelial cells "happy."

Building the menstrual cycle through tissue engineering

Stromal cells are very responsive to progesterone. Even in 2D, progesterone induces a "decidualization response" in healthy endometrial stromal cells, which makes them more like epithelial cells, producing abundant prolactin. Many uterine disorders, not only endometriosis, are characterized by progesterone resistance, in which the stroma cells do not respond properly to progesterone and do not signal the epithelium. This can be assessed in a culture by measuring prolactin production. Different phenotypes are observed when cells are compared in 2D versus 3D, with a much more pronounced production of prolactin taking place in 3D. The phenotypes are maintained much longer in 3D than in 2D.

Epithelial cells have traditionally been difficult to grow, and it is challenging to develop a synthetic 3D matrix that supports both epithelium and the stroma. Research has been done on the gut and the plasticity of stem and progenitor cells, which are also found in the luminal epithelia of the human endometrium. In research involving the gut, a completely synthetic gel was identified that allowed clonal growth of epithelial organoids.

A major challenge in developing an endometrial model for the menstrual cycle with Matrigel or collagen gel is the timescale, because these gels break down. The synthetic gel can be tailored to stay in place for the whole menstrual cycle, allowing the creation of disease phenotypes. Dr. Griffith's lab is adapting these approaches to endometrial lesions, and the microfluidic approaches are being adapted to thermoplastics. Work with the endometrial organoid has now been extended to work with other tissues, such as a pancreatic tumor. A project with Roger D. Kamm, Ph.D., for building microvascular networks and extending them to tumors is also underway.

Dr. Griffith and Dr. Palmer recently got funding to test JNK inhibitors in *in vitro* models.

EVATAR™: The Mother of Microphysiological Systems

Ji-Yong Julie Kim, Ph.D., Susy Y. Hung Research Professor and Professor of Obstetrics and Gynecology (Reproductive Science in Medicine), Feinberg School of Medicine, Northwestern University

The Repro Tissue ChIP 1.0 project started about 9 years ago, led by Dr. Woodruff, an ovary expert who gathered experts on each of the reproductive tissues, including Dr. Kim, with her expertise on the uterus. The team collaborated with engineers from Draper Laboratory to create a microfluidic system for the reproductive tract. The work is being continued with Repro Tissue ChIP 2.0 to study polycystic ovarian syndrome (PCOS), a multi-organ system disease.

Dr. Kim focused her presentation on identifying gaps in knowledge, barriers to advancing the science, and promising approaches and tools needed to further the application of

physiomimetic and organoid models to reproductive health questions. Many barriers and knowledge gaps in reproductive biology remain because of ethical issues associated with research in women of reproductive age and during pregnancy. Experiments cannot be done on these women, so there is a need to study reproductive tissues outside of the body. Although much has been learned from animal models, there are vast species differences; the human model remains the best for human reproduction. Also, hormones are very complex. They are context dependent, fluctuate, and affect the whole body. A platform is needed to deconvolute the complexity while keeping the system intact.

NCATS and DARPA created a mechanism for developing a platform that allows the study of the reproductive tract on a chip. The scalable platform system created enabled all of the tissue compartments involved (e.g., uterus, fallopian tube, follicle, liver, ectocervix, pituitary gland) to communicate with each other. The team worked with Draper Labs to build a platform that would harbor each tissue individually and also allow them to communicate serially through fluid flow. The resulting electromagnetic microfluidic platform, EVATAR™, allows for communication between multiple organs in one platform. The system can be customized for single tissues, two-tissue interactions, and recirculation with up to five tissues.

The driver of the reproductive-tract-on-a-chip is the ovary. The sex hormones produced by the ovary in the menstrual cycle influence all of the downstream tissues. Dr. Woodruff's pioneering work involved stimulating mouse ovaries to secrete estradiol and progesterone over 28 days, similar to a menstrual cycle. Downstream reproductive tissues (human fallopian tube, human recellularized endometrium, engineered human ectocervix, and human liver microtissues) were cultured in 3D and responded to the hormones. EVATAR allows for one universal medium, continuous changing of media, and communication among tissues. The ovary and the 3D cultures responded more robustly in the microfluidic environment compared with static cultures. They "enjoyed being together." The reproductive-tract-on-a-chip can be used to answer research issues that could not be addressed earlier, such as drug testing that involves multiple organs, systematic investigation of disease pathogenesis, personalized medicine, and an understanding of how risk factors affect tissues.

The LATTICE system, a next generation of EVATAR, was designed to mimic each of the different tissues in a more digestible way (i.e., making it easy to use, cost-effective, and compatible with 24-well plate technologies), using materials compatible with hormones and hydrophobic molecules. Initially, some materials that were used to 3D-print the system were toxic to the ovaries, so polystyrene was used instead. The system also needed to have robotic handling capabilities. The LATTICE 8UP culture plate has eight wells and is cost-effective and easy to use. The LATTICE base station is where the microfluidic actuation in the plate takes place, with several motors and sensors. (Dr. Kim presented a video of the LATTICE operation and controls for media flow.) LATTICE was built to fit into a stackable "hotel" that fits into an automated incubator. A robotic arm deposits the 8UP plate in an automatic imager and runs a custom LATTICE imaging profile. The system uses familiar production-quality plates and has high versatility with reliable flow rate control and integration with robotic handling.

Dr. Kim's research has used the LATTICE system to study PCOS, which involves not just the ovary but also metabolic systems and hyperandrogenism. Because PCOS is a multi-organ

disease, little is known about its causality and drivers. The model developed includes the addition of pancreatic islet, liver, and fat steroids. The ovary drives the hormonal components; use of HCG allows the ovary to produce more testosterone. The downstream tissues include the fallopian tube and endometrium, which will respond to the signals received. The system will be used to screen environmental disrupting compounds, such as clinical drugs, per- and polyfluoroalkyl substances (PFASs), bisphenol A (BPA) and bisphenol substitutes, harmful algal bloom toxins, and flame retardants. One drug of interest is metformin, which acts on various levels. Dr. Kim's team is also using LATTICE to explore how obesity increases the risk of endometrial cancer. Some of the early changes associated with neoplasia, such as epigenetic changes, can be explored.

In summary, the development of these models allows researchers to ask new questions, narrow the gaps in knowledge, and break down barriers to advancing science by increasing tissue longevity. In addition to EVATAR and LATTICE, many other platforms are available that allow for robotic handling and minimum human error.

Supplying the Demand for Novel Ligands to Encourage Use of New Physiomimetic Techniques in Achieving Higher Drug Approval Rates in Reproductive Health Therapeutic Development: Three Vignettes

Stephen Palmer, Ph.D., Director, Lead Discovery, Center for Drug Discovery; and Associate Professor, Department of Pathology and Immunology, Baylor College of Medicine

Dr. Palmer presented a review of three drug discovery programs. The activities associated with the drug discovery process include determining the hypothesis, identifying a protein of interest, and early target validation. The process is lengthy, requiring about 3 years before an Investigational New Drug (IND)–enabling study can take place, as well as costly. The developmental costs for TocopheRx, for example, were about $3.2 million.

Development of an FSH receptor allosteric agonist for PCOS

The concept behind the development of an FSH receptor allosteric agonist was the replacement of fertility stimulation injections with orally active agents. This is already happening with gonadotropin-releasing hormone (GnRH) modulators, such as leuprolide, which is no longer expected to be the only agent in this class. Oral GnRH antagonists are already entering the market and expected to move from treatment of endometriosis and fibroids into infertility treatments. The goal of Dr. Palmer's drug discovery process was to identify an agent that addressed FSH and LH excess and met the need for convenience.

Screening for allosteric modulators can reveal both agonists and antagonists. Allosteric modulators can work at very different sites, not only on the extracellular domain that binds the receptor. An emerging theme in G protein–coupled receptors (GPCR) allosteric ligand discovery is that an agonist extends the TM2 to TM7 transmembrane proteins of the GPCR ligand and an antagonist extends the TM3 to TM7 transmembrane proteins. A compound with both FSH and LH activities was desired, because clinicians use a mix of both for fertility treatments. Two molecules that appeared to be good clinical candidates were discovered. TOP5300 has 85% FSH receptor agonist activity and 15% LH receptor activity. TOP5668 is a pure FSH agonist with

100% FSH receptor agonist activity and 0% LH receptor activity. Research by Nataraja et al. showed that increased amounts of TOP5300 met or exceeded FSH's ability to stimulate follicular development. The goal of the preclinical work was to confirm that these small molecules could work in human granulosa cells in a manner similar to that in rodents; the small molecules were actually more effective than FSH. The findings indicated that the small molecules may be working on human FSH receptors in a way that cannot be simulated in a rat model.

In research at Baylor College of Medicine, Dr. Palmer's team looked at differences in patient populations' responses to estrogen production and expression of steroidogenic enzymes. In patients with normal ovarian reserve, FSH was still a more potent agonist in cells. In patients with PCOS, FSH had almost no ability to stimulate estradiol production, whereas TOP5300 retained essentially the same profile seen in patients with normal ovarian reserve. In PCOS, FSH had almost no ability to stimulate StAR and CYP19a1 production, unlike TOP5300. This implied something unique about PCOS and the cells' ability to respond to FSH.

Future opportunities for physiomimetic systems may relate to inflammation, which is a major driver of PCOS pathophysiology, and studies of C-reactive protein (CRP), a key clinical correlate for the presence of inflammation associated with chronic diseases. Pentraxin 3, a ligand in the same family as CRP, has been shown to be elevated in patients with PCOS. Studying inflammation created by immune cells, particularly macrophages that affect cells from patients with PCOS, can lead to an important understanding of how small molecules and proteins differ.

Development of a JNK inhibitor for endometriosis

Endometriosis occurs in a hormone-dependent environment that is surround by inflammatory events, so hormonal and inflammatory modulation are key targets for treating the disease, reducing pain, and correcting infertility. The peripheral nervous system is also involved in sensing the migration of cells toward endometriotic lesions and communicating through pain. JNK inhibitors are believed to influence both of these systems. JNK1 and JNK2 are part of controlling endometrial cells, with some contribution from JNK3. Similarly, in immune cells, JNK1 and JNK2 are the dominant regulators. In modulating pain, JNK3 is dominant and expressed in the peripheral nervous system. In the liver, inhibition of JNK2 is greater than that of JNK1, causing a compensatory increase of JNK1 expression in the liver, leading to adverse events.

Two JNK1 inhibitors have been studied: bentamapimod and tanzisertib. Phase II clinical trials of bentamapimod in endometriosis were less than optimal, mainly because the progestin response was variable among patients. Also, medroxyprogesterone acetate (MPA) was used as the placebo (because of the need to treat the pain) and introduced significant variation that prevented identification of a clinical benefit. Tanzisertib was studied in idiopathic pulmonary fibrosis, which has some overlaps with endometriosis. Tanzisertib's JNK1 inhibition was weaker than its JNK2 inhibition and may have contributed to elevations in liver transaminases. Although there was some clinical benefit, the toxicology prevented the research with tanzisertib from going forward. Interestingly, the compound would never have gone on to clinical trials if it had showed toxicity in preclinical models. The presence of inflammation in humans appears to change how the liver responds to JNK inhibitors.

Preclinical work on bentamapimod was done by Dr. Osteen and Kaylon Bruner-Tran, Ph.D., at Vanderbilt University. Addition of the JNK inhibitor resulted in remarkable suppression of matrix metalloproteinase-3 (MMP-3) and MMP-7. When lower concentrations (which were marginally effective in the presence of increasing progestin concentrations) were used, there was remarkable suppression of MMP-3 and MMP-7. This indicated that the JNK inhibitors might be able to reverse the insensitivity to progesterone that occurs during the course of the disease. The work was done by using a surgically induced rat model of endometriosis. The key observation was that the JNK inhibitor at a relatively high dose caused the absence of c-Jun expression (i.e., immune cells invading the endometrium were no longer recruited), and no phospho-c-Jun was detected compared with antide or control. Apoptosis induced by antide caused significant apoptotic events in the endometrial and immune cells. The immune cells characterized by CD45 were reduced with the JNK inhibitor, compared with either antide or endometriotic lesions. In the ipsilateral horn in this rat model, there was a decrease in both interleukin-12 (IL-12) and IL-10, which are both associated with endometriosis. In the contralateral horn, the JNK inhibitor had no impact on expression of those cytokines. Findings were similar with monocyte chemoattractant protein-1 (MCP-1). A similar type of JNK inhibitor response was evident in immune cells taken from patients with relapsing-remitting multiple sclerosis. In summary, with bentamapimod, c-Jun phosphorylation in ectopic lesions was suppressed about 150-fold, confirming on-target engagement of JNK. Bentamapimod caused regressions with fewer lesions than in women who received MPA. Also, immune cell–derived cytokines were reduced below study start, while they increased with MPA. In the eutopic endometrium, bentamapimod showed no impact on JNK phosphorylation. Indirectly, immune cell–derived cytokines were reduced, reflecting impact from the periphery on the eutopic endometrium. There was no change in ovarian function or irregular menstrual cycle.

Bentamapimod was deprioritized compared with another agent whose clinical path was observed more easily. The researchers' goal now is to develop an even better JNK inhibitor, with similar selectivity, better activity, and no discrepancy between JNK1 and JNK2. This is being done using DNA-encoded chemical libraries, which allows for preparation of billions of compounds in small volumes. Proteins of interest can be incubated with DNA-encoded libraries, and then polymerase chain reaction can be used to identify compounds that bind to the target. One example involves work that identified a potent thrombin inhibitor. Dr. Palmer's lab is using this hybrid approach to identify compounds that will meet the criteria for JNK1, JNK2, and JNK3 inhibition and achieve novelty.

Another approach to studying the activity of kinase inhibitors in cells is to look at cells in menstrual effluent, which is known to have both endometrial cells and immune cells relevant to the activity of kinase inhibitors. Dr. Palmer is current working with NextGen Jane, a company that has developed a diagnostic based on collecting menstrual blood from tampons. The researchers have been able to characterize various cell systems and the cells from which they can be derived. These cells can be established in culture and may lead to a preferred profile of JNK inhibitors in menstrual cells and demonstrate relevant immune cells.

Regulation of iron in anemia of chronic disease

Inflammation is believed to affect hepatic cells and bone marrow–derived macrophage regulation in iron balance. If macrophages' ability to import iron is modified, there is an opportunity to regulate the signals that the macrophages bring into the ovary. Dr. Palmer's team is beginning to look at this system by observing the response of hepcidin to the addition of BMP2 and tacrolimus. The findings to date appear robust, and there are opportunities to further understand impacts on macrophage behavior and hepatic cell responses to inflammation.

Conclusion

The recent emergence of oral GnRH antagonists has raised the visibility of opportunities in private-sector investment into women's health. Having more women in leadership roles has also increased the emphasis on reproductive health diseases. Targeting GPCRs is possible as new technologies are emerging to address them. New selectivity among kinase inhibitors in parallel indications increases the likelihood that the development of safe and effective nonhormonal therapeutics is possible. Protein degraders are another new approach in drug discovery, and more work is needed in this area.

Uterine Biology and Pathophysiology Speaker Q&A

Moderator: Candace Tingen, Ph.D., Program Official, Gynecologic Health and Disease Branch, NICHD

Q: Dr. Tingen asked Dr. Tagle to discuss his partnerships with pharma, how they work day to day, and whether chips developed through the program are currently being used by pharma for drug testing.

A: Dr. Tagle said that NCATS has interacted with pharma on an individual basis, which tends to be time-consuming, but it is more efficient to work with the IQ Consortium, which is made up of companies that share common research and development interests. Working with the consortium streamlined the process and centralized the communication lines. In terms of day-to-day work, the companies are actively involved in organizing workshops, writing manuscripts, and providing guidance documents for industry use of microbiological systems. Johnson & Johnson, Pfizer, Merck, Roche, and AstraZeneca are using commercialized platforms that are already available through some startup and spinoff companies. Pharma also collaborates directly with developers to bring in platforms of interest.

Q: Diana Monsivais, Ph.D. (Baylor College of Medicine), asked Dr. Griffith how the hydrogel culture conditions that she uses in her endometrial organoid systems compare with what was previously published by Boretto et al. and Turco et al. She also asked whether these culture conditions are appropriate for studying differentiation and regenerative processes of the endometrium.

A: Dr. Griffith said that the other researchers used Matrigel, which is the canonical organoid culture format and includes components such as growth factor and other matrix proteins that are not in the culture that she uses. Matrigel has a slightly greater efficiency in allowing

organoids to emerge from single cells, but it degrades quickly, making studying the entire menstrual cycle difficult. Dr. Griffith uses a synthetic gel that allows for tailoring of the degradation rate, so the whole culture can remain stable over a 28-day cycle. Although her data for the endometrium have not yet been published, published data for pancreatic tumors go into detail comparing the *in vivo* matrix and microenvironment to the *in vitro* model. Her culture is not identical to Matrigel in the efficiency for initial emergence, but other features, such as reproducibility, are better.

Q: Dr. Monsivais asked Dr. Kim whether the LATTICE system can be used in endometriosis models to determine endometrial interactions with immune cells or other relevant outcomes in endometriosis, such as pain or hormone synthesis.

A: Dr. Kim said that LATTICE is a tool that provides an eight-well plate connected by microfluidic channels. She has not studied immune cells in the fluidic system herself, but she noted that Dr. Griffith has worked with immune cells that flow through the channels, so this is something that LATTICE can do. Dr. Griffith said that her lab has done cocultures of macrophages with endothelial tissue and that differentiating and combining the immune cells in a stable manner is challenging. Her colleagues are using the cocultures to study macrophages and are making observations in the presence of sex hormones that are not typically seen in standard macrophage culture. Dr. Griffith has not yet started working with circulating immune cells for the endometrium. She said that much work is emerging on immune cells in different organ systems and that the protocols must be reviewed very carefully. She will submit a paper on her work in about 6 weeks.

Q: Lisa Halvorson, M.D., asked Dr. Palmer to speak more about the strengths or limitations of his *in vitro* methodology as applied to either PCOS or endometriosis.

A: Dr. Palmer said that when advancing a drug, some simple mechanistic assays need to be run before going on to complex mechanisms. Simple systems should demonstrate an on-target anticipated response; if not, something is wrong. Currently, none of the cellular assays are adequate to predict efficacy of a substance in PCOS or endometriosis. Reproductive diseases generally involve an inflammatory response, so there is a need to be aware of the relevant immune cells that are modulating endometrial and other cells.

Q: Passley R. Hargrove-Grimes, Ph.D. (NCATS), noted that Dr. Griffith is moving away from polydimethylsiloxane (PDMS) and toward thermoplastics, and she asked about the major issues that need to be surmounted when using thermoplastics, in addition to cost.

A: Dr. Griffith said that a major factor to consider is that PDMS is highly oxygen permeable and most thermoplastics are relatively impermeable; the delivery of nutrients with thermoplastics must be redesigned, because it cannot depend on the diffusion of oxygen. Also, various kinds of fabrication tools are needed for bonding the layers together with thermoplastics. This can be

expensive, but there are many companies that will do custom fabrications. Dr. Griffith also noted that PDMS is not easy to manufacture at scale.

Q: Dr. Tingen asked Dr. Kim whether the endometrium in the PCOS LATTICE model is still the same recellularized sample or a different sample.

A: Dr. Kim said her PCOS LATTICE system uses endometrial organoids but not the decellularized part. The epithelial cells did not survive well in the decellularized system.

Q: Virginia Chu Cheung, Ph.D. (University of California, San Diego), asked Dr. Kim whether the LATTICE system is compatible with organoid/spheroid cultures and whether it is validated with monolayer cultures.

A: Dr. Kim said that her LATTICE system has Transwell compartments for 3D tissue cultures. All of her systems are 3D; she does not work with monolayers.

Q: Dr. Hyun noted that Dr. Kim said that mouse ovarian tissue was used instead of human tissue, because using the latter would be unethical. He asked why this would be unethical and whether the system would enable the *in vitro* maturation of human eggs if human ovarian tissue were used.

A: Dr. Kim said that she was referring to bench science and discovery research, which should not use ovaries from women of reproductive age for experiments. She was not referring to *in vitro* maturation for a patient.

Q: Stuart Moss, Ph.D. (NICHD), asked the panelists whether the presence of semen should be considered in these systems.

A: Drs. Griffith and Kim said that the presence of semen would not apply to studies of endometriosis and PCOS. Dr. Kim said that the answer would depend on the research question. For example, looking at fallopian tube cilia in the presence of semen would be a doable experiment.

Q: Dr. Osteen said that much of his work has focused on early-life environmental influences on the risk of developing endometriosis. He asked about the potential for using immunotherapy with good immune cells to target the disease.

A: Dr. Palmer said that in his JNK inhibitor work, it was amazing to see that the lesions could be influenced with no effect on the eutopic endometrium. He said that Erin Greaves, Ph.D., is working on distinguishing the differences between immune cells in the eutopic environment and those in the periphery. Characterizing the two types of cells and seeing how therapies affect them could be an objective. A goal could be to affect the peripheral cells that are driving

the lesion but not affect the eutopic cells so that pregnancy could take place without adverse events.

Q: Suzanne E. Fenton, Ph.D. (National Institute of Environmental Health Sciences), said that the development of the systems seems to be driven by pharma companies that need to know how to assess a compound for treating a disease. She asked how much of the effort is going into disease prevention or the effects of environmental chemicals on the menstrual cycle and the risk for diseases like PCOS or endometriosis.

A: Dr. Kim said that the bulk of her research involves looking at disease initiation and the effects of chronic stressors on benign, non-diseased tissue, as well as the changes in different cell types at the molecular level, including epigenetics. This may lead to treatments that prevent those changes from occurring. Dr. Kim is also working with iPSCs to differentiate cells of interest and identify changes that take place in women with the disease.

Listening Session

Moderator: Candace Tingen, Ph.D., Program Official, Gynecologic Health and Disease Branch, NICHD

Dr. Hyun asked whether there were plans to construct a model of the male reproductive system. Dr. Kim said that Dr. Woodruff and her graduate student published some work in this area (the "dude cube") but are currently not continuing due to the lack of personnel and funds. Dr. Kim agreed that research in this area should be pursued.

Asgi Fazleabas, Ph.D. (Michigan State University), asked Dr. Palmer how the DNA libraries and billions of compounds are narrowed down to look for biological function. Dr. Palmer said that there is a cheminformatics process to look for common chemical elements that appear in information from a DNA-encoded library. An example in his presentation showed a 90-fold enrichment for JNK compared with other chemical signatures. The goal is to identify the most highly enriched compounds, resynthesize them, and demonstrate that the chemical produced exhibits the anticipated activity. Once the chemical "playground" is identified, the researcher can narrow it down to find the chemical entity with the selectivity sought. The added benefit, particularly when working with kinases, is the ability to identify whether the emerging chemical signature for a particular kinase is represented across 10 or 20 other kinases, indicating a selective compound.

All attendees were asked to comment on reproducibility, its importance, and how an urgency can be created for replication of some of these systems. Dr. Griffith said that although not all researchers are doing experiments with the same patient samples, reagents and protocols need to be replicated. For the endometrium, several researchers have come together to create standard protocols and reagents. Much of Dr. Griffith's work on the synthetic matrix has been driven by reproducibility. The matrix is easy to use and not costly. A lab in England followed a protocol from her lab and was able to create the matrix exactly. Part of the issue is determining

the protocols that everyone agrees with. Devices are more difficult and costly to replicate, but at least some of the tissue engineering components may be reproducible.

Dr. Kim said that the MPS-Db is important. It is a centralized system where all data can be entered so that other researchers can see and replicate the work. Dr. Kim is also working with primary tissues from patients, so there is much variability. However, some of the variability disappears when cells are placed in a certain environment and react. There is still variability, but less than with just taking the tissue and analyzing it.

Dr. Palmer said that a common industry practice to ensure that a discovery is reproducible is to record everything about a discovery in a notebook and hand the materials to another scientist in the same lab or a different lab. Following the procedure and getting the same results is a nice internal quality control practice to ensure that the methods described are easily transferred.

Dr. Hargrove-Grimes said that NCATS is encouraging the IQ Consortium or the IQ MPS Affiliate, which represents about 30 different pharmaceutical companies, to put their data in the database. Their reticence is due to their lawyers' advice and the risk of entering proprietary data. The Tissue Chip Testing Centers are taking the devices developed, doing a technology transfer, and trying to reproduce exactly what is done in the developers' labs. This work will help build confidence in the technology if a different lab in a different space can replicate the findings. One of the testing centers recently published a paper showing that the major source of variability and lack of reproducibility is in the cells that are used. Any type of primary tissue will be difficult to recapitulate in other labs, so NCATS is trying to encourage the use of PSCs, which can be kept alive for an extended period, allowing for movement among labs. But primary tissue is needed for studies of some diseases, particularly PCOS.

Attendees were asked to address areas where nonprimary tissues can be used and the particular applications that require that type of tissue. Dr. Osteen said that he did not think that very complex systems are an efficient screening tool. There are ways to screen with target cells that are more reproducible than a multicellular system. When doing the initial discovery, a researcher may not want to have the most complex organ-on-a-chip or organoid system to screen. Complexity can be brought in while trying to mirror the likelihood of an adverse event in a Phase II trial. Part of the answer is to use the cells that allow for screening and discovery and then to keep checking the findings in more complex systems. In toxicology studies, for example, a complex system would be expensive. The models can be used as appropriate while moving up the chain toward a human trial.

Brandy Heckman-Stoddard, Ph.D., M.P.H. (NCI), asked Dr. Kim about variability in her system using other organ systems as well as endometrial tissue. Dr. Kim said there is definitely variability in the *in vitro* organoids. When taking cells out of the body and subjecting them to the same media, the response is not as variable as when taking just tissue and looking at its heterogeneity. There are commercial sources of tissue and some lines that are derived from human tissues. Variability will always be there; it is just a matter of doing sufficient replicates to get more robust data.

Dr. Heckman-Stoddard asked how often diverse populations are considered in these types of organ systems. Dr. Kim said that researchers need to be aware of and adequately represent the differences in populations. Other projects at her institution are looking at race differences in gynecologic disease and cancers. Covering the different diverse populations will likely come as a result of collaborations with different centers; one institution will not have all that is needed.

Dr. Tingen asked Dr. Hyun to comment on bioethical issues related to race, ethnicity, and class when building models for endometriosis and other disease categories. Dr. Hyun said that there are several confounding issues related to bioethics. For example, there is a lot of debate about whether to even use the concept of race in biomedical research; much is socially constructed and may not be helpful from a biological viewpoint. Another issue involves reproducibility and how to validate physiomimetic models well enough to provide confidence that they are recapitulating real human biological events and not merely artifacts, especially if these systems are being used for new discoveries. Philosophically, there is room for creeping doubt. Are the systems representing human biology for only a subset of a sample? The issue touches on the quality of the science and the conclusions drawn and how the systems will be used going forward. Dr. Hyun said that the issue has also come up with organoid projects. The first question scientists will get from industry is about how to know that the model is representative. To make the comparison, the scientists need to use data or fetal tissue, which raises other political and ethical issues. Validation and ensuring that all of the modeling is accurate are evergreen issues.

Dr. Palmer said that one of the most intense validation efforts he has seen is associated with toxicology models, in which the toxicology response in a culture system is linked with reported toxicology observed in *in vivo* animal models. When they differ, the model is limited to addressing a particular toxicology. Many predictive toxicology models have been developed over the past 20 years but are generally not embraced by industry because of fear of missing something. Industry opts for *in vivo* models.

Dr. Cheung commented on the use of iPSCs, noting that as models get established with them, there is greater access to diversity. She said that a major question relates to determining the gold standard and suggested that all the sequencing efforts and advances allow for at least establishing and referencing the model system. Once the healthy baseline of a strong endometrial cell is set, complexity can be built by modeling the disease.

Dr. Kent asked whether the discussion of variability refers to variability within technical replicates or within biological replicates. Dr. Hargrove-Grimes said that it refers mainly to biological replicates.

The attendees were asked what researchers should aim to accomplish with the uterine models within the next 5 years. Dr. Palmer said that he would like to determine four or five

factors that are representative of progesterone insensitivity in an endometriotic system and have confirmation in a cell system and patient samples. This is currently not well defined and is an important aspect of treating patients.

Dr. Fazleabas said he would like to address why ectopic tissue creates an environment in which a subset of patients are infertile. The lesions could be the same in all patients who have lesions, but only a subset will have an infertility phenotype. Another question relates to how ectopic tissue alters the uterine environment and makes it difficult for these patients to get pregnant. Dr. Fazleabas said that he and Dr. Kim discussed using her LATTICE model to possibly identify some of the components involved. Immune cells add to the complexity. A recent study was published on a subset of macrophages in the endometrium that contributes to lesion development.

Dr. Griffith said that part of the issue is that every patient is different. When a disease involves 10% of women, there will be subgroups. One challenge is the lack of large population studies with enough revealing measurements in the protocols. The work being done with menstrual effluent and immune cells, for example, is inconsistent, because the methods used each have their own limitations. Building models *in vitro* should account for the calibration and the spectrum of *in vivo* phenotypes. Not all endometriosis patients have the same problem with getting pregnant. The next steps are to understand the different patient populations, regardless of the model used, and build a model to look at recruitment of circulating immune cells into an existing lesion. The foundation is still thin.

Dr. Fazleabas agreed, saying that methods for defining the disease, much like that for defining breast cancer, and for determining changes that could then be grouped to model subgroups of patients are needed. This would involve a large-scale approach and large cohorts.

The moderator noted that complex questions are being asked of complex systems. Are there simpler questions to ask—for example, about normal endometrium alone? Dr. Griffith said that the myometrium and junctional zone are not well understood. A patient with adenomyosis may not have lesions on imaging, but there are definite differences in the myometrium.

Dr. Shikanov asked whether anyone was working on the vaginal microbiome, noting that the vaginal and gut bacteria (microbiome) contribute to pathological conditions. She asked whether adding immune cells to the model would also require adding the bacteria. Dr. Griffith said that this model is difficult, because zero oxygen is needed, and it is difficult to get a continuous anaerobic microenvironment. Her lab is starting to work with the colon microbiome, and she would like to work on the gut microbiome with the liver and endometrium. Connecting the gut microbiome to something else is complicated and costly.

Dr. Hyun asked whether there are ways to factor in environmental factors (e.g., stress, social determinants of health) in the physiomimetic model systems. Dr. Kim said that she is doing some race disparity studies in uterine fibroids and endometrial cancer and seeing biological

differences. However, they may be not inherent biological differences but rather the result of chronic stressors. Dr. Kim said the endpoint differences are there, but it is difficult to delineate the cause without looking at the genotype or variants.

Dr. Osteen said that there is a higher incidence of preterm birth in African American populations and that different populations need to be used in the models to understand race-related issues. His group has a pending grant to look at endometriosis and the risk of preterm birth and is building chip models that stress the maternal–fetal interface. When that interface is stressed, there are immunological consequences for the fetus, whether or not there is preterm birth. Transgenerational studies cannot be done on a chip currently, but there may be ways to do tandem designs that use the models to ask questions about how stress on one system affects the fetal system.

Dr. Griffith noted the gene–environment interaction and that cells from a patient have lived through the life of the patient. She asked whether it is possible to really replicate the patient's situation with iPSC-derived tissue. Dr. Kim agreed, noting that genetics and epigenetics play large roles. She said that she is definitely seeing differences in the iPSCs, depending on the patient's demographics and the disease. When somatic cells are reprogrammed, they become pluripotent and very young. This may offer an opportunity to look at the stressed cell and reprogramming it to see what is different in the cell. Much work has been done with the iPSCs, especially in epigenetics. Some researchers cite an "epigenetic memory," but the meaning is not clear and could be explored further.

The attendees were asked to discuss partnerships, how to start them, and who needs to be on the teams. Dr. Hargrove-Grimes said that Dr. Tagle has been working with forming partnerships over the last 10 years and that developers need to be brought together with regulatory officials and pharmaceutical companies. Anyone interested in this field should get in touch with Dr. Tagle. Also, NCATS has started working with the Standards Coordinating Body for Gene, Cell, and Regenerative Medicines and Cell-Based Drug Discovery to create standards for tissue chips. The field of research is very small and intertwined, and the researchers interact with each other often. NCATS would like to have more people join consortium meetings. Working together in a cooperative and collaborative manner will help advance the work.

Dr. Palmer said that the collaborations with pharma will not start with large pharmaceutical companies. The best option is to find small pharma partners (i.e., startups that are hungry for a system to help them advance).

All attendees were asked to discuss scalability and give their thoughts about uterine models that may be scalable for therapeutic development; screening is controversial. Dr. Griffith said that she is not dismissing screening, but there is a need for higher-level information.

Dr. Palmer said that the whole point of screening is to confirm the target engagement. The right target must be confirmed before going to more complex biological systems.

Attendees were invited to give any additional comments for colleagues or NIH. Dr. Griffith said that getting NIH funding in this area is difficult. About 80% of NIH's extramural funding is for investigator-initiated research. For gynecology, only about 30% to 40% is; everything else goes through Special Emphasis Panels (SEPs). It is hard to build a community going through SEPs, because there are no standing study sections. The community stays small because of bottlenecks in funding and institutional barriers to bringing together people with expertise in this area. Dr. Griffith said that most of her work has been funded by philanthropy or DARPA. Dr. Tingen said she was not sure whether the NIH funding percentage cited by Dr. Griffith was true for NICHD gynecology.

Dr. Halvorson said that NICHD will be using the input from this meeting to discuss the future of reproductive health research at the institute. NICHD was asked to look further at the use of model systems and to participate in developing the strategic plan. The goal is to continue to show leadership in this area so that funders are enthused about broadening the technologies and range of investigators, with less dependence on Requests for Applications (RFAs).

Dr. Harris said much focus has been on the female reproductive system and not the male one. Her team is making organoids from the epididymis, which is being studied by only a few groups. The questions raised at the meeting about accessibility of tissues and reproducibility were relevant to her area.

Dr. Hyun said that in bioethics, there is a call to engage patients more in clinical trial design. He asked whether there was a need for patient engagement in either the design of the models or their clinical use. Dr. Hargrove-Grimes said that for the clinical trials on the chip initiative, a section in the RFA required teams to work with patient advocacy groups. It is not possible to move forward scientifically without incorporating the viewpoints of patients struggling with diseases. For example, a team working on progeria is incorporating input from the world's largest progeria foundation.

Dr. Shikanov agreed that working with patients and understanding their needs opens new approaches in research. Her team is working with patient feedback in studies of endocrine function preservation in pediatric cancer survivors and fertility preservation in transgender patients.

Attendees were asked if any groups are working on models that address specific ages or different reproductive states. Dr. Osteen said that the different agencies need to talk with each other more and link investigators together when cross-communication would be beneficial to patients. There is currently no forum for this.

Dr. Griffith said that in endometriosis, the immune and hormonal systems differ in adolescents and in 30-year-olds. Average hormone concentrations change as a function of age, and her team is starting to work with *in vitro* models to mimic this. In almost all of the literature on

endometriosis, women are in their mid- to late 30s. Now younger patients are being seen, and samples are being collected from them.

Dr. Duncan said that her team is studying ovarian aging. The ovaries are among the first organs to age, and their impact on systemic aging and vice versa are not well understood. The models can connect multiple tissues to study the mechanisms of aging and how the reproductive tract can drive some of the aging processes. Dr. Duncan's team is about to start a project that will apply the LATTICE system to look at cellular senescence in terms of communicating across organs.

Dr. Palmer said that Celmatix is also focusing on ovarian aging. The company is currently not employing an *in vitro* model system but may be interested.