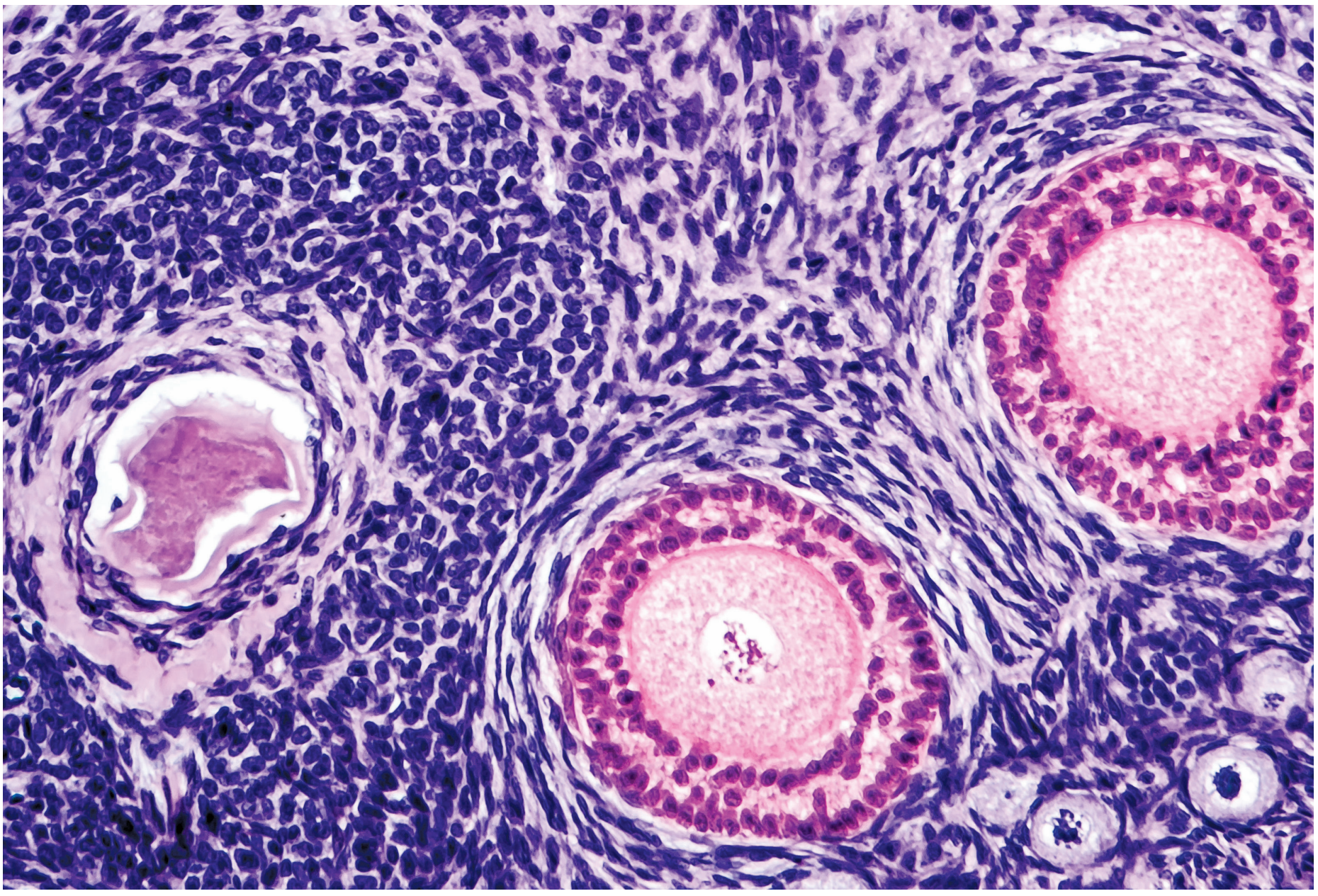
Engineering Fertility Options for Cancer Survivors

Aiming to preserve fertility for women undergoing cancer treatments, researchers are developing biomaterials that mimic the structure and function of the ovary.



Follicles move toward the interior of the ovary and expand as they mature.

BY SARAH ANDERSON, PHD

FROM THE MOMENT A WOMAN is born, her body is prepared to one day give birth to children of her own. Women come into the world with about one million follicles, the clusters of cells containing an immature egg, in their ovaries. "It's from that million-follicle pool that, during the reproductive lifecycle after puberty, follicles then are recruited so that ultimately only about 400 ever ovulate," said Teresa Woodruff, a reproductive biologist and biomedical engineer at Michigan State University. "My interest originally was to understand how follicles make decisions to be the follicle that's ovulated when a woman is 19 and it's May, while one sitting right next to it won't make that same journey for maybe 10 or 20 or 30 years," she said.

Woodruff's career took a journey of its own. While working as the basic science director of a cancer center, she heard about a young boy with cancer who came in to store his sperm, safeguarding his chance at conceiving a child should the cancer treatment affect his fertility. "I thought, 'Oh, that's amazing! What do we do for our young women?' And the physician who told me about this said, 'Oh, we shouldn't worry about that; they need to really focus on their cancer.'" But the one million follicles a woman is born with is the most she'll ever have, and if they're damaged, they don't regenerate. "A light bulb went off partly from an equity issue," Woodruff said. "I really started thinking about how the work I was doing in ovarian follicle biology could be translated into helping with fertility."

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> *– Teresa Woodruff, Michigan State University*

Since that fateful day, Woodruff coined the term and pioneered the field of oncofertility, or managing fertility for cancer patients for whom the toxicity of chemotherapy and radiation is not constrained to cancer cells (1). A major focus of her research has been developing systems where follicles can mature outside of the natural ovary. She has inspired a new generation of researchers who continue to bioengineer materials that mimic the ovarian environment and use them for new applications, developing *in vitro* and *in vivo* follicle development platforms for fertility preservation and preventative toxicity analysis. Their goal is that one day, all cancer patients may truly be able to focus only on their cancer because their concerns about fertility have been addressed rather than ignored.

Preventative measures

The follicle is a three-dimensional structure comprised of the oocyte (the immature egg) surrounded by granulosa and theca cells, which provide physical support and biochemical signaling that help the oocyte mature. "When you put [the follicle] on a flat surface, it flattens out, so you lose this three-dimensional structure. And when you lose the three-dimensional structure, you lose the contact between the cells," said Ariella Shikanov, a biomedical engineer at the University of Michigan. "This is super important because granulosa cells are all attached to each other. And they also have tight junctions, tiny channels between the granulosa cells and the oocyte that allow them to nurture and feed the oocyte."

To enable the follicle to maintain its

critical three-dimensional architecture, Woodruff and collaborator Lonnie Shea, both at Northwestern University at the time, turned to alginate, a polysaccharide derived from algae used in a number of bioengineering applications (2,3). They developed an alginate-based hydrogel material that encapsulates the follicle like a piece of fruit suspended in Jello. This "reductionist approach," which provides the physical support the follicle needs to develop without influencing its biological interactions, comes with advantages, said Francesca Duncan, a reproductive biologist at Northwestern University. "You can remove the follicle from the context of the ovary in the body and say if I add a certain supplement, how is the follicle responding to that without thinking about the complexity of the whole body and the systemic environment. So, it's a very controlled model system to study follicular development," she said.

Shuo Xiao, a reproductive biologist and toxicologist at Rutgers University, has relied on the alginate hydrogel to study how cancer drugs affect follicle maturation. His team adds drug compounds to the cell culture media, which penetrate and diffuse through the hydrogel to reach the encapsulated mouse follicle. They then acquire microscope images of the follicles to see how many survived and measure their diameters over time to track growth. They also measure the concentration of secreted hormones that indicate effects on follicle health and ovulation, monitor the ovulation process in which the follicle ruptures to release the egg, and assess the size and quality of the egg. The researchers collect follicles that show signs of toxicity for further genetic and proteomic analysis to gain insight into the mechanism and validate the effects of the corresponding drug in an animal model.

CREDIT: ARIELLA SHIKANOV/UNIVERSITY OF MICHIGAN

***Ariella Shikanov engineers tunable synthetic hydrogels that support follicle development* in vitro *and* in vivo.**

By screening cancer drugs using this platform, the team has found that chemotherapy drugs that work by damaging DNA can impair follicle development. "Granulosa cells are highly proliferative *in vitro* and naturally or physiologically, so they are a very sensitive target," Xiao said. Drugs that regulate hormone activity used for diseases such as breast cancer can also have side effects. "If the drug can target estrogen receptors or progesterone receptors, they can also target the ovary and cause abnormal hormone secretion and ovulation," Xiao said. The researchers have also uncovered valuable trends in drug toxicity, including that checkpoint kinase inhibitors, which are used alongside DNA-damaging agents to block a DNA repair pathway in cancer cells, are more harmful to follicles when they target checkpoint kinase 1 (4).

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>
> *– Ariella Shikanov, University of Michigan*

Xiao hopes that his work will equip oncologists and cancer patients to make better informed decisions about treatment. "We can provide them the information and also what is the underlying mechanism, so in that way, they do not only know is it possibly toxic or not, they also know why," he said. "Our research can enable us to find another unique pathway in the ovary so we can target molecules in that pathway to develop a drug. It won't affect the efficacy of the cancer drug, but at the same time, it can protect the ovary."

Dynamic design

For now, many women who need to undergo chemotherapy or radiation treatment are seeking ways to preserve their fertility. One possibility is to retrieve and cryopreserve their eggs, which could later be used for *in vitro* fertilization. However, there are several groups who cannot take advantage of this option. The process of maturing the eggs inside the body takes at least one month, and people with aggressive forms of cancer often cannot wait that long to begin treatment. The process also requires injecting estrogen, which can drive hormone-responsive cancers such as breast cancer. And in prepubescent girls, the ovary will not produce mature eggs.

Shikanov wants to provide alternatives for these patient populations. She envisions that an individual could have an ovary removed prior to treatment, providing a source of healthy follicles that could be grown into mature eggs in the lab rather than in the body. To do so, she is developing biomaterials that mimic the dynamic

Researchers implanted a biomimetic ovarian scaffold seeded with follicles into a sterilized mouse, enabling her to give birth to pups.

ovarian environment to support *in vitro* follicle development.

Human follicles balloon as they develop, growing approximately 600 times their size. Dormant follicles reside near the exterior of the ovary, where the tissue is stiffer, and migrate toward the interior, which is softer, as they grow. Researchers realized that this change in rigidity plays a critical role in follicle expansion and maturation. Woodruff's team demonstrated that a two-stage culture system in which follicles were first encapsulated in the alginate hydrogel and then removed from the hydrogel better mimicked the shift in stiffness in the natural environment, yielding mature human eggs for the first time (5).

"Understanding the dynamics and understanding the architecture is really important to be able to then mirror that in a culture system," said Duncan, who trained in Woodruff's lab. "We were doing it sort of forcefully, where you're in alginate, or you're not in alginate, or we're changing the concentration. But it would be much better to create a system where whatever the products are that the follicles are producing as they grow, that's communicating with the biomaterial and breaking it down in that time-dependent way that makes sense with follicular growth."

Toward this end, Shikanov is engineering follicle-responsive polyethylene glycol (PEG)-based hydrogels to support follicle development over time. Her team first incorporated degradable peptide crosslinkers to accommodate the follicle's massive expansion (6). "Encapsulated follicles secrete proteases that break down those crosslinkers and open up the space, still staying in the three-dimensional environment, but allowing softening of the material around it," Shikanov said. The follicle also secretes extracellular matrix proteins as it grows. To mimic the natural extracellular matrix deposition process, the researchers attached additional peptides to the hydrogel that bind to and sequester the proteins (7). "Because PEG is a synthetic material, you have the freedom to make it tunable," Shikanov said. "Every time, we add another step, making it more and more biomimetic."

Shikanov is also exploring ways to recapitulate the biochemical signaling that promotes follicle development. While follicles depend on hormones such as follicle stimulating hormone and luteinizing hormone to fully mature, researchers have discovered that local signaling between cells in the follicular environment influences their early stages of growth. Woodruff's team encapsulated early follicles in the alginate hydrogel and observed that the follicles showed better growth and survival when cultured in groups rather than individually (8). "The more that you have growing together, the happier these follicles are," Duncan said. "That told us that there's probably some factors that these small follicles are producing."

Shikanov's team wondered if they could achieve a similar effect by encapsulating adipose-derived stem cells with the follicle inside their hydrogel. These stem cells secrete cytokines such as vascular endothelial growth factor that are thought to be involved in follicle development. The researchers found that coencapsulating the follicles with adipose-derived stem cells improved their survival, growth, and maturation (9,10). "You can recapitulate and reintroduce this continuous crosstalk between the follicle and other cells that are found in the ovary normally," Shikanov said. She imagines that adipose-derived stem cells, one of the few sources of stem cells left in the adult body, could be easily accessed from the patient's fat and used to support the development of her follicles *in vitro*. Shikanov's team is also using the alginate hydrogel to systematically profile the follicle transcriptome and secretome, aiming to identify a cytokine cocktail that could be added to *in vitro* culture systems to promote follicle growth (11,12).

Solutions that last

Another fertility preservation option for cancer patients is to have a portion of ovarian tissue collected and cryopreserved prior to treatment. This tissue is then transplanted onto the remaining ovary after treatment and recovery. While the procedure overcomes many of the limitations of egg cryopreservation, the stored ovarian tissue may contain cancer cells if the patient had a metastatic or blood-borne form of cancer. "When you cryopreserve tissue, there is a risk that you will be reintroducing cancer cells, and you don't want to take this risk with the patients," Shikanov said.

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> *– Monica Laronda, Northwestern University/Ann & Robert H. Lurie Children's Hospital of Chicago*

CREDIT: JANICE B. TERRY/ANN & ROBERT H. LURIE CHILDREN'S HOSPITAL OF CHICAGO

Monica Laronda studies the dynamic structure of the natural ovary and leverages the spatial control of 3D printing to create a bioprosthetic organ.

Even for cancers that don't present this risk, ovarian tissue transplantation may not be ideal. Clinicians have found that in transplanted tissue, more follicles are activated from the dormant state to the growing state than usual. However, normally only one of these follicles will ovulate each month, and the rest are lost. This increased activation depletes the follicle reserve and limits the lifespan of the tissue graft to approximately two to five years.

While researchers aren't sure what causes the transplanted follicles to burn out so rapidly, this phenomenon may be related to changes in the structure of the remaining ovary due to cancer treatment. Duncan recalled a presentation she saw about a woman who had her ovarian tissue collected, underwent chemotherapy, and then received the tissue transplant when she was in her mid-30s. "Her residual ovary where they did the transplant essentially looked like a postmenopausal ovary. And I couldn't help when I saw that image but to think, 'Oh my gosh, we're taking this healthy tissue that was frozen, and we're now transplanting it back onto a postmenopausal-like ovary, which we've shown in our work is fibrotic, stiff, and inflammatory,'" Duncan said. "We also know that the longevity of these grafts that have thousands of follicles are not forever. And part of the reason might be that we're not transplanting them back into a hospitable environment."

To address these shortcomings, researchers are developing biomaterials that could stand in for ovarian tissue. Follicles that are free of cancer cells could be isolated from the surrounding cryopreserved tissue and placed in an artificial scaffold. The scaffold would be implanted to support follicle growth inside the body where the complex biochemical signaling that promotes early

CREDIT: MICHIGAN STATE UNIVERSITY

Teresa Woodruff established the field of oncofertility to address reproductive challenges for people with cancer.

follicle development would occur naturally. The follicle secretes growth factors that recruit tissue and blood vessel cells that gradually replace the scaffold. "If you just create the appropriate environment for these follicles to do their thing, they will recreate that ovarian tissue in the way that needs to happen, which is beautiful," said Monica Laronda, a reproductive and regenerative medicine researcher at Northwestern University and Ann & Robert H. Lurie Children's Hospital of Chicago.

To create this type of scaffold, "we use similar materials that can support follicle growth, but then go away once the whole process of remodeling and regeneration has completed," Shikanov said. Her team once more tweaked the design of their protease-degraded PEG hydrogel, adding an adhesive peptide that binds to endothelial cells to support blood vessel formation (13). The researchers tested their follicle-encapsulated hydrogel in mice where the ovaries had been removed and found that the scaffold attracted blood vessels and supported the development of the follicles throughout the entire life cycle. They also observed that the majority of the follicles remained in the dormant reserve 60 days after implantation, suggesting that the dynamic hydrogel environment can recapitulate the mysterious process in which dormant follicles are selectively activated to grow in the healthy ovary. "What [Shikanov] has done is to really engineer back in some of the context of the ovary," Woodruff said. "You start to reassemble the biological cues so that over the long haul, you can have an ovary that functions for decades."

Shikanov is further finetuning her follicle-growing hydrogels for both *in vitro* and *in vivo* applications with the goal of "trying to find as many and as diverse fertility preservation options as possible," she said.

Leaving an imprint

Researchers have also explored 3D printing as a technique to create materials that support the growth of isolated follicles in the body. "Every ovary has this kind of soft tissue skeleton that is the extracellular matrix," Woodruff said. Guided by the structure of the ovary's natural "skeleton," researchers have used 3D printing to create scaffolds with well-defined pores that each house a follicle. The open pores enable nutrients, oxygen, and signaling molecules to easily reach the follicles and permit blood vessel infiltration. Contact between the follicle and the edge of the pore provides the physical support required to maintain the follicle's three-dimensional shape while allowing it ample space to expand.

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> *– Francesca Duncan, Northwestern University*

Researchers have found that some pore designs support follicle development better than others. "I was really fascinated by how can we change the architecture of the microenvironment of a cell to be able to make that biological influence, and 3D printing was really a great tool to be able to change specific parameters in a reproducible way," said Ramille Shah, a biomedical engineer and cofounder and chief scientific officer of the biomaterials company Dimension Inx.

In a landmark *Nature Communications* study, Shah, Laronda, Woodruff, and colleagues reported that pores with narrower corner angles enabled more points of contact with the follicle, improving its survival (14). Using their optimized design, the researchers 3D printed a scaffold, seeded it with follicles expressing green fluorescent protein (GFP), and implanted it in mice where ovaries had been removed. They then mated the mice so that they became pregnant and gave birth to pups. When the researchers examined the pups using goggles containing a filter that excites GFP, they saw that their whole bodies glowed green, confirming that they came from the follicles in the scaffold.

"I will never forget that day when I saw the first litter born and they were GFP-positive," Laronda said. "We had the idea that it would work because we were getting eggs ovulating from the scaffold in a dish, but to actually be able to transplant it and have the mouse mate and then wait weeks and weeks and weeks before you actually see a pup that's born — I was elated!"

The study reported the first functional 3D-printed soft organ. Laronda and Shah are now interested in using 3D printing to spatially pattern a material with different rigidities, mimicking the physical gradient in the natural ovary that maintains the reserve of dormant, or quiescent, follicles. "If we are going to reconstruct the whole ovary with these different regions, 3D printing would enable us to engineer the different environments that the quiescent follicle pool needs, but also that the growing follicles need," Laronda said.

Shah is testing different materials to identify those that would be viable in a human implant product. "It needs to be biocompatible, and we want it to degrade over time and get replaced by the natural tissue that normally cells see. That limits the foreign body response if it can be remodeled and vascularized," she said.

The scaffold that gave rise to the green glowing mouse pups was made of gelatin, which is derived from collagen, one of the proteins in the ovarian extracellular matrix. While it meets many of these criteria, the need to rely on a natural source to synthesize it and the fact that its ability to be 3D printed is sensitive to temperature and humidity do not make it the best choice moving forward, according to Shah. "It's even more important to understand not [only] could it work functionally, but can it

Chemotherapy and radiation can impair ovarian function, necessitating new innovations in fertility preservation.

be translated and can it be manufactured in a scalable way?" Shah said.

Laronda is studying the proteins in the extracellular matrix of bovine ovaries, which are more abundant than human ovaries, to better define their identities and roles in the development of the follicle from its initial primordial state (15). "We're trying to understand the extracellular matrix components and how they're organized and how different proteins within that material that exists outside of the follicle can influence follicle activation," she said. "Some of those proteins in particular, we think, may be controlling the rate of primordial follicle activation. And really, that would dictate the functionality and the longevity of a transplant."

Extracellular matrix proteins from bovine ovaries may provide a source, or (more likely) a source of inspiration, for materials used in a next-generation 3D-printed ovary. "We're going to answer a lot of fundamental questions by figuring out the environment," Woodruff said. "Natural sources are necessary to identify where we're going, but in the end, I think it will be a synthetic source."

The need for a more complete roadmap to inform efforts to recapitulate the ovary applies not only to the extracellular matrix, but to the entire organ. "In most tissues, we're able to say, here are all the cell type components of a tissue. And in the ovary, we've been really behind the eight ball," Duncan said. She attributes this lag to the historical lack of technology such as single cell analysis and the challenge of amassing comprehensive data for an organ that is constantly changing. "In order to get the best quality egg and the most robust system, we have to be able to engineer this complexity back in, and the only way we're going to do that is to be able to understand every part of the ovary, like the cell types, their dynamics, and the matrix that comprises it," she said.

With this goal in mind, Laronda is participating in the Human BioMolecular Atlas Program (HuBMAP) to create an atlas of the human ovary (16). "We're looking at single cell level transcripts and molecular mechanisms, which includes proteins and mRNA, and neighborhood relations of one cell to another to map the organ in a very defined way," she said. "Our project is to take this information and reverse engineer this environment into something that would be useful as a bioprosthetic ovary."

Laronda's role as codirector of the fertility preservation program at Lurie Children's Hospital has helped her appreciate the difference her research can make. "Approximately 20 percent of the patients that we see each year come from other hospitals. And so, it's that important to them that they'll travel, even as they're going through their cancer treatment, to take advantage of what we can offer for fertility preservation," she said. "Seeing those young patients now gives me the hope and the drive to work over the next many years before they come back to need that tissue." ■

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