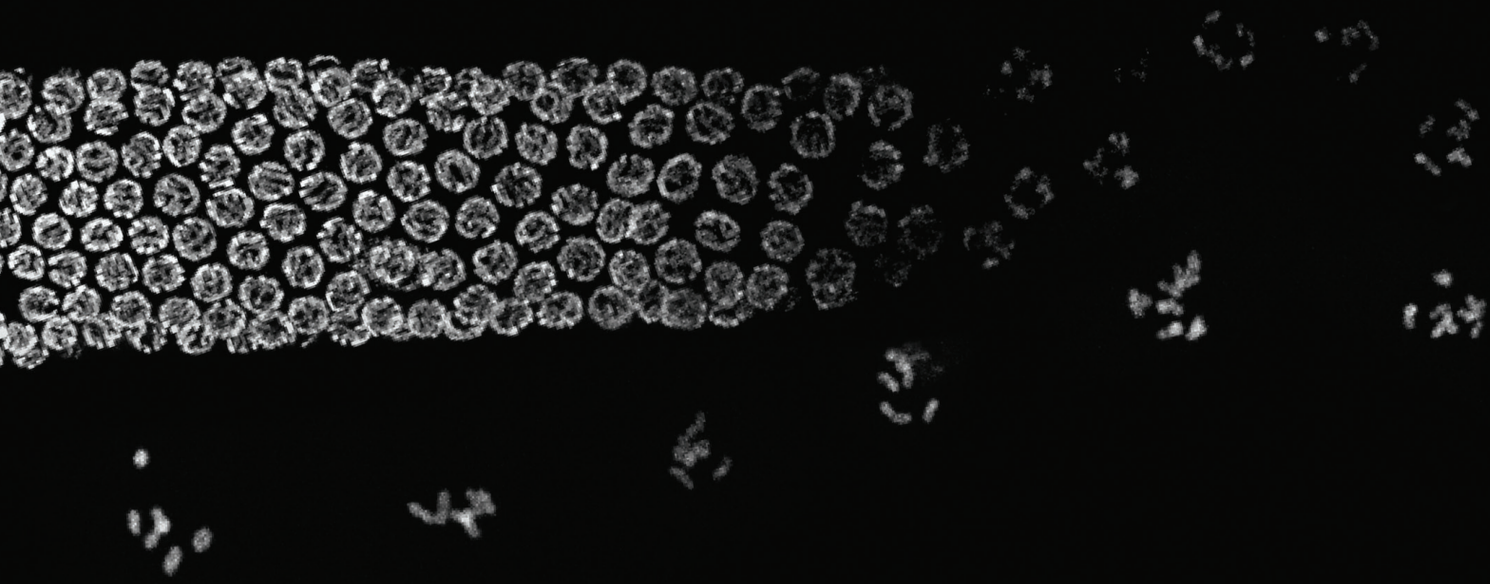


EMPTY NESTS

When a woman is pregnant with a baby girl, the pair is at an incredible, understudied biological juncture: Mom, baby, and potential grandbabies are all in one vessel—flourishing on or suffering from the same nourishments, reacting to one another, interacting with the world around them. Past, present, and future nestled into one body; somehow three beings at once.

Perhaps your grandma had a set of those Russian nesting dolls—a round, squat woman who houses another squat woman, who contains yet another decorative matryoshka doll, and so on, until you reach a teeny version of all the women before her on the inside. When made in their traditional way, these dolls—also called babushka, or grandmother, dolls—are hand carved and decorated with a unifying theme. Each doll shell is made from the same block of wood so that they whittle similarly and warp together over time.



Primitive worm, preliminary model. The gonad of *C. elegans* is orderly, visible, and relatively simple compared to humans' undercover reproductive tracts. Meiosis—the strictly regulated process by which a cell divides to become a gamete—may hold secrets to causes of infertility, and worms are helping Pitt researchers expose the genetic generation of generations.

All images courtesy Judith Yanowitz

THE BIOLOGY BEHIND INFERTILITY

BY ROBYN K. COGGINS

Likewise, ovaries—and the eggs, or oocytes, inside them—are influenced by the body they're in, the body they've been in.

"The ovaries themselves are affected by the environment," explains Aleksandar Rajkovic, who's an MD/PhD professor of obstetrics, gynecology, and reproductive sciences and the Marcus Allen Hogge Professor in Reproductive Sciences at the University of Pittsburgh studying the genetic markers of ovarian aging and dysfunction. However, he says, "It's difficult to study the connection between the ovary environment and the overall health of an individual, since ovaries are not easily accessible in a woman to actually study." Most studies before the genomic revolution relied on animal models or postmortem dissection and didn't give a complete picture of living ovarian function.

Another method of assessing ovarian and reproductive health is looking at families. Some of Rajkovic's research focuses on large cohorts of women and their female relatives to spot trends. The problem, however, is that by definition, families with fertility issues are small.

Consider the condition known as primary ovarian insufficiency, or POI. An irreversible spectrum disorder of the ovaries, POI causes subfertility or infertility. Sometimes its root is genetic; in other cases, POI is triggered by autoimmune disorders like lupus or by chemotherapy damage to the organ. Of the 1 to 4 percent of American women who have POI, only about 10 percent of cases have a known cause, usually a mutation in the FMR1 gene, which is also linked to fragile X syndrome.

When ovarian function dwindles, hot flashes, mood swings, low libido, vaginal dryness, missed periods, and many of the other symptoms associated with low estrogen and menopause flare. Half of the women who spontaneously present with these symptoms see at least three doctors before any lab work is done or diagnosis is made. POI is often missed and is a greatly misunderstood condition.

But POI is not simply early menopause. Imagine being told that your ovaries are failures. POI used to be called premature ovarian failure, evoking some kind of personal biological bankruptcy. Imagine being 27 years old—the average age of onset—and getting the news that you'll likely never have a family without reproductive assistance. Maybe you didn't want children in the first place, but that quick snip of fate's thread can still seem cruel. Yes, you will probably hit menopause early. While your friends are having children, you'll look at their swelling bellies and wonder why your body won't mindlessly jumpstart its opaque reproductive machinery.

And, until very recently, women were often given the news of this diagnosis over the phone, or even by e-mail.

"It's not uncommon for them to be at work," says alum Lawrence Nelson (MD '73), who's a commissioned officer in the U.S. Public Health Service and studies the genetic origins of POI in mouse models and in humans. "Then they have to find a place to cry."

In his private practice, Nelson saw his first case of POI in the '80s—"I'd never heard of it before," he says. He looked for more information in the medical literature, but not much was written on POI, which meant he had very little to offer his patients as they sought to understand their condition.

Not surprisingly, one of Nelson's more recent studies on the psychosocial effects of POI found that the top three words women used to describe how they felt after diagnosis were "devastated," "shocked," and "confused." Physicians like Nelson and Rajkovic are out to change that upsetting experience. But, first, they need to know more about the condition.

In 1990, when Nelson got a chance to do research at the National Institutes of Health, he pounced on the opportunity to dig into POI's origins. His basic science approach led to the discovery of Mater—a gene, which, when knocked out in mice, stops embryonic development in the oocytes and causes sterility. He's now translating that to clinical research, examining the exomes of women with POI to find more causes.

Nelson suspects the cause will be more complicated than just finding a gene that leads to POI. The condition is often unpredictable—ovarian function may wax and wane, which allows some 5 to 10 percent of women with the diagnosis to conceive. As of yet, there's no reliable way to tell whether that will happen in a particular woman.

He speculates that POI's cause will be more like congenital deafness and other conditions with multiple genetic origins and biologic interactions. That's why researchers are turning to large-scale sequencing of the genome and the exome (just the protein-coding genes).

Furthermore, POI can affect more than just fertility, Rajkovic explains.

"Women who have premature menopause or ovarian failure—which usually means when they stop menstruating prior to age 40—they are at risk for osteoporosis, for cardiovascular mortality and morbidity, and overall mortality is actually increased in these women. And it's not been well understood what causes this increase. . . . Ovaries are so essential to women's health."

Although hormone replacement therapy delivered through a skin patch can mitigate some POI symptoms, its effects on this population aren't well studied, and HRT doesn't address infertility at all.

As Pitt's director of reproductive genetics, Rajkovic, with his nine-person lab team, is trying to fill in some of the knowledge gaps surrounding ovarian development and dysfunction. Recently, the crew sequenced genetic samples from families with strong evidence of genetic causes for ovarian dysfunction. "This is an international effort. We've collaborated with individuals in Saudi Arabia and in Turkey to actually recruit families that had genetic forms of ovarian failure. And we then used the new genetic sequencing approaches to . . . identify causes."

Funded by a $2.5 million R01 grant from the Eunice Kennedy Shriver National Institute of Child Health and Human Development, Rajkovic and his team discovered two genes that had never been previously implicated in POI: MCM8 and MCM9.

"In the first three families that we actually sequenced, we found that in one family there was a mutation in MCM8, and in two families there was a mutation in MCM9—MCM9 is MCM8's partner." In women with MCM8 and MCM9 mutations, their ovaries do not develop typically, and inside are very few or even no germ cells; plus, chromosome breaks abound. Using their findings on this pair, Rajkovic's lab members hope to model more genes implicated in infertility.

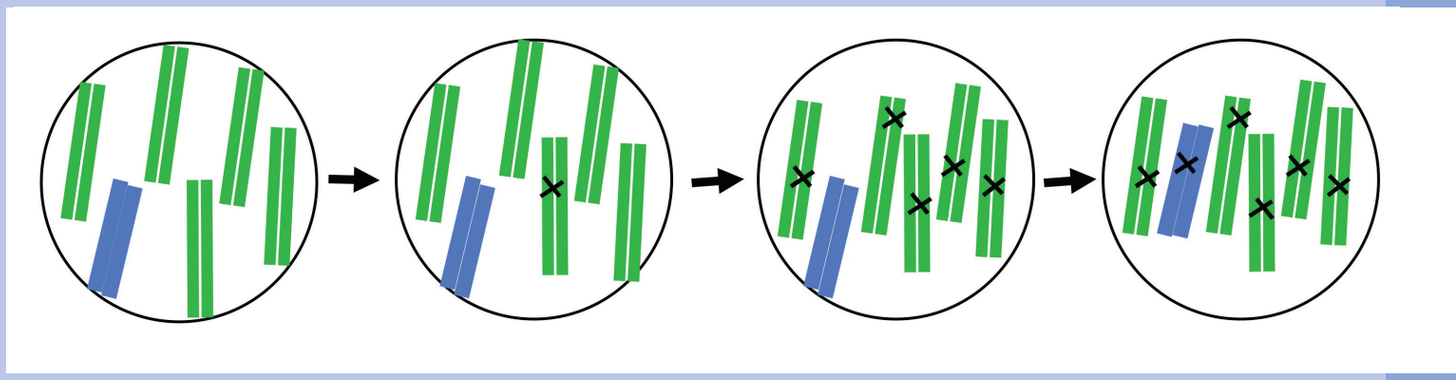
In total, they are sequencing nearly 100 POI cases and 300 typical menopause controls. In animal models, they've found more than 300 genes implicated in ovarian insufficiency, which they've termed the "ovariome."

Rajkovic, along with postdoctoral fellow Michelle Wood-Trageser (PhD '09) and colleagues from pathology, chemistry, and human genetics at Pitt, has continued investigating 10 of these ovariome genes, and the scientists are currently recruiting patients for further studies.

In addition to the ovary, his lab also studies uterine fibroid tumors, testicular failure, and problems in spermatogonia (undifferentiated germ cells that eventually become sperm).

Others at Pitt, including Rajkovic's Magee-Womens Research Institute colleague Alexander Yatsenko, an MD/PhD, are looking at genetic mutations on the X chromosome that cause male infertility. (Recall that men have XY sex chromosomes, whereas women have the more redundant, and sometimes protective, XX.) Yatsenko, an assistant professor of obstetrics, gynecology, and reproductive sciences, and collaborators in Poland found that the TEX11 gene, inherited from a man's mother, interferes with meiosis, producing genetic errors in the cell and, ultimately, causing male infertility. Those results were corroborated by colleagues in Germany, and their findings were published in *The New England Journal of Medicine* this spring.

In both men and women, elevated levels of follicle-stimulating hormone (FSH)—a substance that kick-starts production of sperm in men and initiates follicular growth, and thereby ovulation, in women, among other biological duties—can signal fertility issues, as well. Doctors sometimes use FSH levels as an indicator for ovarian



As mom's and dad's DNA begins to mingle, the cell needs to monitor the swap, kind of like a border agent. Yanowitz has developed a theoretical framework, pictured here, of these crossover "checkpoints" during meiosis—points where the cell checks its papers to make sure everything's in order as the two genetic bits become one. Once the first crossover (x) is made, a checkpoint is activated.

reserves: too much, and you're in premature menopause; too little, and you might have polycystic ovarian syndrome (another possible cause of infertility). Abnormal hormone levels plus a few missed periods usually prompt doctors to look for POI, but that alone is too imprecise to yield a definitive diagnosis. Furthermore, researchers suspect that problems with the follicles themselves, either through dysfunction or depletion, might contribute to POI, but no one is sure yet.

An April paper by Rajkovic and colleagues on FSH receptors in two Indian sisters with POI showed some incredibly specific causes of the syndrome—an inherited recessive trait that resulted in a novel variation of FSH receptors, which affected protein signals in the women's bodies, triggering POI.

Siblings from consanguineous parents, as in the Indian case, are much more common elsewhere—more than 10 percent of marriages are between first cousins in some parts of Asia and the Middle East. These close ties are ideal for studying POI because genetic mutations are easier to detect. But, according to Rajkovic, "For us to really understand ovarian function, we need to understand development." In other words, he wants to know what goes on in early germ cells. And he wants to understand what precise genetic roots rob many women of their dreams of having families.

"Part of who we are is to reproduce," Rajkovic says. "People don't understand the anguish and depression and effect on relationships and marriages. It's profound."

According to him, ovaries and their function are a window to future generations, or a lack thereof.

And "oogenesis, the formation of the fully competent egg, begins in the ovary!" he adds. "If eggs have abnormal chromosomes, we try to understand the mechanisms," Rajkovic says. So to study ovaries, you'll want to know about eggs; and to study the eggs, you'll want to know their ovarian origin's origin—meiosis.

Aristotle also wondered about conception and development. That mighty philosopher used an observational technique still replicated in contemporary high school biology; he would ever so gently chip a small hole in a chick's shell, careful not to penetrate the membrane underneath. He hoped to watch the development of the creature inside—to see how it went from yolky mass to chirping chick. How did the heart form? When did the beak appear? And can the inner life of a chicken's egg give hints about human development?

Today, reproductive researchers go primitive—seeking answers from a wriggling, transparent creature known as *Caenorhabditis elegans.*

Pitt's Judith Yanowitz is a PhD assistant professor of obstetrics, gynecology, and reproductive sciences. She studies meiosis in *C. elegans*—tiny nematodes that are an ideal subject for genetic tinkering and studies on fertility, as they're simple in structure and relatively straightforward in development. The worms have just six pairs of chromosomes and 100 million base pairs (compared to 23 and 3 billion, respectively, in humans), which made them an attractive subject during early whole genome sequencing. (Theirs was the first whole genome to be sequenced.) *C. elegans*'s reproductive tracts, especially, are orderly: From end to end, the gonad's germ cells and developing embryos form a progressive timeline of conception to birth.

Yanowitz explains this as she swaps dishes of *C. elegans* under a microscope—she's looking for just the right squirming millimeter to illustrate her work. She and the half-dozen or so colleagues in her Magee-Womens Research Institute lab have been engineering mutant *C. elegans*, which are typically self-fertile hermaphrodites with XX sex chromosomes, for genetic variations and processes related to reproduction. A portion of their research focuses on the rare males, who have only one sex chromosome, represented as "X0," and thus further remove a genetic variable. Broadly speaking, Yanowitz wants to figure out where errors in cell division begin. That means wending her way back to meiosis—one of the earliest events of life for any sexually reproducing creature.

Yanowitz has dark curly hair, a standing desk, and the most enthusiastic attitude toward worms you've ever seen. In her guest editor's introduction to a special issue of *Methods* last year, Yanowitz suggested that *C. elegans* isn't just a toolbox—it's a toolshed, and one that needs expanding.

"They're cool!" Yanowitz says of the worms. "Let's go look at them."

Under the microscope, she can see the *C. elegans* wriggle in their dishes, glowing green and pink according to their mutations. Their eggs, little time capsules, line up inside the slippery sine wave of a body. Her lab techs have fixed the worms at various stages of reproduction, allowing observation through the entire process. Each batch of worms has a different genotype, crafted by the crew, to play out controlled iterations of chromosomal exchange.

Several times, Yanowitz exclaims, "I'm in love with chromosomes!"—how they copy, how they express in different regions, how DNA gets repaired, how it all coordinates. "There are many questions about the underlying biology that just knowing the genes doesn't reveal."

One of the key events during meiosis is a genetic swap: "The maternal and the paternal copies of every chromosome in our cells actually exchange DNA; that's a process known as crossing over, or recombination," Yanowitz explains. In diagrams, recombination looks somewhat like 46 people playing a game of Twister—legs and arms wrap around each other and tangle together. But this game of Twister is twisted: The arms and legs, hands and feet, actually swap between partners, resulting in genetic diversity in the offspring's DNA. Some places are more prone to swapping than others, and these are called hotspots. "One of the projects in the lab is trying to understand what makes a hotspot hot, what calls up this particular region, what makes it more prone for this double-strand break," Yanowitz says.

Yanowitz's lab studies mutations that affect double-strand breaks and how they're made. That is, why some chromosomes get breaks and others do not.

Sometimes things go awry. "If you fail to get a crossover, or if it happens in what we call 'unfavorable' places on the chromosome—we don't really understand what that means—the chromosomes mis-segregate during cell division." That can mean offspring with extra or missing chromosomes. Or it can mean a failure for the chromosomes to ever create offspring—to cross the next "checkpoint" of meiosis.

The concept of checkpoints is new, and even a little controversial, in biology and genetics. During the two-part process of meiosis, Yanowitz and others theorize, the burgeoning cell checks up on its own development, much like a passport check at an international border—somebody has to make sure everything is in order. If not, the journey's over.

One gene they've identified is xnd-1, which regulates chromosome crossover and segregation. Experiments conducted by postdoctoral fellow Mainpal Rana, a PhD, have shown that its absence can cause sterility in *C. elegans*. So, too, can genes nos-1 and nos-2. Ninety-eight percent of worms missing xnd-1, nos-1, and nos-2 are infertile, and 92 percent have no germ cells. Their findings on xnd-1 were published in *Nature* in 2010.

Similarly, through studies of *C. elegans*, Yanowitz's team found that mutations in him-5 reduce the frequency of crossover on the X chromosome and change crossover distribution.

More recent studies have provided further evidence for this genetic border patrol: "We have evidence that there actually is a true crossover checkpoint that monitors each chromosome pair, each maternal-paternal homolog pair, to make sure that they've created this crossover," Yanowitz says. Those results were submitted for publication this fall.

But let's back up. Meiosis occurs early in fetal development, and its result is a package of gametes—the eggs or sperm needed for future reproduction. After DNA replicates, meiosis begins, then the twisted chromosomes swap bits of themselves at hotspots and line up along the poles of a cell. Spindle fibers draw pairs from mom and dad to each other, at which point they scuttle over to either end of the cell. Finally, a cleavage forms to clip off both sides into their own haploid cell, called daughter cells. Each daughter cell has a nucleus and a single set of unpaired chromosomes called chromatids.

At this stage in female development, meiosis pauses for about 7 million oocytes (immature egg cells). In female humans, hormones like FSH won't shoot the starter pistol for the million or so oocytes remaining at birth to trigger what's known as meiosis II until puberty.

For males, meiosis I and II begin in puberty and run on autopilot, ready for reproductive action—though sperm, too, need to cross checkpoints through meiosis on their way to maturity. In mouse models, Rajkovic's lab has discovered another gene pair, SOHLH1 and SOHLH2, that, when knocked out of the genome, stop sperm from maturing.

Supposing a gamete does mature, in meiosis II, a very similar process to meiosis I occurs, though chromosomes don't replicate again. The chromatids split, align, and dance across the cell to opposite ends. Then chromatids become full-blown chromosomes and break into a total of four new cells. In males, all four become sperm; in females, two or three become "polar bodies," which often die or assist in other bodily processes, and the lucky one begins the journey to mature ovum.

The oocyte begins its maturation pilgrimage to the follicles and eventually to ovulation. In all, only a few hundred eggs will make this trip before menopause. (That voyage, too, is incredibly complex and ongoing. Many eggs are maturing at once, competing for the right to attempt fertilization—and that maturation process takes over a year for each. And that's before the thing has even met sperm—which, as we know, bring their own genetic baggage to the relationship. It's a wonder any of us are even born, when you think about it.) And, like any odyssey, a flat tire or wrong turn of the caravan can delay arrival at one's final destination.

"Aleks and I talk quite a bit about [how] *Wouldn't it be great if we could detect bad oocytes?*" Yanowitz says. "That's the million-dollar question: Is there something fundamentally different or some signal that an oocyte that has not correctly undergone meiosis is sending out?"

"Chromosomal abnormalities play an important role in an egg's demise, but some [eggs] that survive will give rise to offspring with unbalanced chromosomes," Rajkovic adds. So a discovery at one stage will probably help elucidate problems at another developmental stage.

How might genetic knots and splinters warp future generations? The answers to that question go beyond just infertility and into the realm of aging and DNA repair. Eggs, and to a lesser extent sperm, carry proteins, a mess of mitochondria, and other essential building blocks of life.

By whittling away at the causes of POI and other variations in the reproductive system, researchers could eventually identify infertility before it happens. Rajkovic dreams of a future where artificial ovaries stave off early menopause and help women conceive without an egg donor.

For now, scientists will have to settle for returning to the beginning, again and again.

As Rajkovic identifies mutations for POI, Yanowitz would like to generate homologous mutations in *C. elegans*. Then, she'll evaluate the functional significance of those variations to see whether they cause fertility or meiosis defects in the worms.

"This will allow for a rapid screen of putative pathogenic variants that we identify in our sequencing of patients," Rajkovic says.

When Rajkovic's team published the papers on MCM8 and MCM9, Yanowitz says, "One of the first things Aleks said was, 'You should study it in worms!' I immediately looked it up online and said, 'Unfortunately, of that whole complex, those are the two proteins that are not conserved in worms and flies.'"

So, they're back to the beginning, brainstorming potential collaborations, ready to start anew. ■