Learning to Knock out Male Infertility

Infertility worries many would-be parents. For men, such concerns may become even more of an issue because male infertility might be increasing. According to Mitch Eddy, head of the gamete biology section of the Laboratory of Reproductive and Developmental Toxicology at the NIEHS, infertility is a major problem in the United States. "Something in the neighborhood of 2.5 million couples of reproductive age are infertile," he says, "and as much as half of that infertility is due to the male." Unraveling the causes behind the rising rate of male infertility requires knowledge about the genetic and environmental factors that affect the development and function of the male contribution to reproduction—sperm. In some infertile men, sperm may be faulty for hereditary reasons, and in other men, environmental factors may cause infertility. Eddy explores the genetic pathways that control sperm development to better understand the process and to determine specific environmental agents that might affect it.

Before searching for what can go wrong in sperm development, investigators must first understand the process that, unhindered, creates millions of normal sperm on a daily basis. Sperm begin as primordial germ cells—undifferentiated cells in an embryo. During spermatogenesis, germ cells divide through mitosis and then meiosis to produce four spermatids, which carry half of a normal cell's complement of DNA. Over a two-week period, the spermatids develop into mature sperm, which consist of a head that contains the genetic "payload" and a tail, or flagellum, that uses the power of adenosine triphosphate (ATP) to propel the sperm to an egg. A structure called an acrosome covers the front of each sperm's head and contains enzymes that help the sperm penetrate the egg. Eddy calls a mature sperm "a little package of DNA with a motor on it. Its whole purpose is to deliver that package of DNA to the oocyte, to allow initiation of development of a new organism."

Building and operating such a specialized structure as a sperm requires the help of specialized genes, ones that get turned on only to produce the proteins needed for developing and driving sperm toward fertilization. One such protein occurs during glycolysis, or the breakdown of glucose to make ATP. An enzyme called glyceraldehyde-3-phosphate dehydrogenase (GAPD) is used about halfway through the pathway of glycolysis, but sperm use a unique version of the protein known as GAPD-S. Using antibodies developed against unique portions of GAPD-S, Eddy and his colleagues found that this protein does not show up until a sperm matures, and then only in the flagellum. In fact, the GAPD-S enzyme does not appear to get turned on until sperm prepare to fertilize an egg. Apparently, the enzyme acts as a "turbocharger" for the sperm—giving it the ATP it needs to power the high-speed beating of its flagellar motor.

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(bottom, left to right) Eugenia H. Goulding, Seok Kwon, Donna O. Bunch, Paula R. Brown. (top, left to right) Kiyoshi Miki, Dahai Zhu, E.M. Eddy, William D. Willis.

Gene KO

To really understand sperm development and function, however, Eddy must define the genetic pathway and the crucial characters that orchestrate those processes. He pursues those goals by selectively eliminating the function of individual genes that are involved and then looking for what happens or fails to happen. This process is called the gene knock-out technique, and in Eddy's laboratory it begins with embryonic stem cells from brown mice. Eddy picks a gene to knock out and then replaces it with a modified gene, fitted with a mutation that keeps the replacement gene from working properly. An electrical jolt drives the modified gene into the embryonic stem cell to replace the normal gene. The cells containing the modified gene go to Gina Goulding, a biologist in Eddy's laboratory, who injects about 15 of them into black-mouse blastocysts. "After I inject the cells into the blastocyst," Goulding says, "the whole thing collapses, and it looks like somebody sat on it. But then, if you leave it in an incubator for two or three hours, it reexpands and looks like you've never touched it." Then, Goulding implants the blastocyst in a mouse host that acts as a surrogate and gives birth to the mouse that develops from the blastocyst. If the cells containing the modified gene were incorporated into the blastocyst, some of the resulting mouse's hair will be light in color. Goulding explains that mice carrying the modified gene "can be anything from a black mouse with just a little bit of brown around the face to one that is almost completely gray with big white patches. And we see everything in between—sometimes they even appear polka-dotted or striped." Whatever the color, the mice with more light-colored hair carry more of the cells containing the modified gene.

Finally, breeding the mice that carry the modified gene in a portion of their cells can produce some offspring that carry only the modified gene, so that the normal gene has been completely knocked out. Although the knock-out technique gives

E.M. Eddy

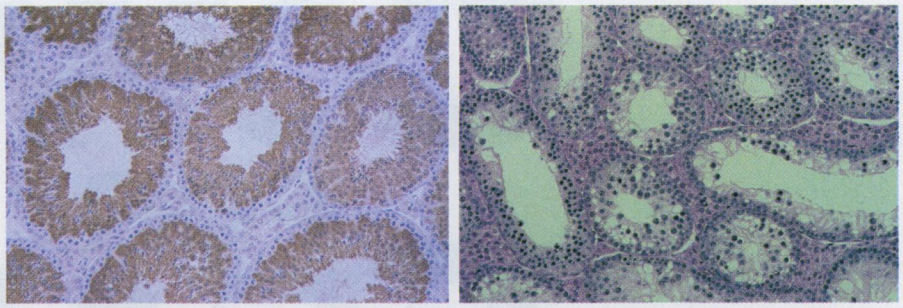
The tell-tale spot. Mice in which critical genes have been knocked out, resulting in light-colored spots of fur, are used by Eddy's group to study various aspects of spermatogenesis.

Eddy and his colleagues the opportunity to dissect the genetic control behind the development of sperm, he warns, "There are lots and lots of genes involved, and you can't study them all. The trick is to try to pick out some that are important in this process."

One intriguing possibility for selecting crucial genes involves heat. Organisms from bacteria to humans have so-called heat-shock proteins. These proteins were first discovered in fruit flies and appeared when the flies experienced increasing temperatures. Investigators speculate that these proteins somehow protect cells from the heat. One of a heat-shock protein's most important roles involves its work as a "chaperone," meaning that it helps a forming protein fold into the appropriate three-dimensional configuration that is required for the protein's proper functioning.

But why study these proteins in sperm? Spermatogenesis in mammals happens to be very sensitive to heat and requires an environment that is 5–7°C below body temperature. Most mammals achieve this optimal temperature by keeping the male gonads outside of the body cavity and by using a heat exchanger-like apparatus that cools blood going into the gonads by transferring the heat to the blood leaving the gonads. Eddy speculated that, because other mechanisms had evolved to protect spermatogenesis from the effects of heat without the help of heat-shock proteins, those proteins might have acquired other functions.

Following that hunch, Eddy and David Dix, then a postdoctoral fellow in Eddy's laboratory and now a research biologist at the EPA in Research Triangle Park, North Carolina, selected a heat-shock protein called HSP70-2 that is found only in spermatogenic cells and knocked out its gene. Without that gene—and, consequently, without its protein—spermatogenesis begins in a mouse, but then stops and the cells die before they become spermatids. Maybe, Eddy thought, HSP70-2 plays a crucial chaperone role. Without it, important proteins might never acquire their normal function and spermatogenic cell development would stop. Further experimentation revealed that knocking out the gene for HSP70-2 stops spermatogenic cell development at a specific stage of the cell cycle called the G_2/M transition, just before the first division of the chromosomes in meiosis. Making that transition requires the work of a protein called Cdc2 that has protein kinase activity, which means that it adds phosphates to other proteins, turning some on and others off.



E.M. Eddy

Stoplight for sperm. A histological stain of testis cells from a normal mouse (left) shows the presence of HSP70-2 from early meiosis through the postmeiotic phase. A stain (right) of testis cells from a mouse in which the *Hsp70-2* gene has been knocked out shows no HSP70-2 and no postmeiotic spermatids or spermatozoa.

But to make Cdc2 work, it must be combined with another protein called cyclin B1. Eddy says, "This is a theme that nature has used over and over again, where you have two or more components that interact to produce the functional end subunit."

When Eddy and his postdoctoral fellow Dahai Zhu examined Cdc2 in spermatogenic cells of mice in which the gene for HSP70-2 had been knocked out, they found that Cdc2 was not bound to cyclin B1 and it lacked kinase activity. However, when Zhu made a homogenate of the testes from a mouse in which the gene for HSP70-2 had been knocked out, and then added HSP70-2, Cdc2 bound to cyclin B1 and acquired kinase activity. That result indicated that HSP70-2 must be a chaperone that binds to Cdc2, allowing it to fold properly so that it can interact with cyclin B1. In explaining that work, Eddy says, "It's not only the components of the cell; it's how they're put together and how they're regulated that is extremely important."

Fruitful Applications

Eddy expects that his work may be applied in many ways. For one thing, knowing how sperm develop normally points out how they might be damaged by toxins. "We used to have a fairly naïve idea," Eddy says, "that a chemical would come in and bind to a particular protein and that protein wouldn't allow the cell to function. We thought of a few proteins as being likely targets. Now that we know that there are many essential protein–protein interactions, we realize that there are a very large number of ways that cell function can be interfered with by environmental chemicals." For example, an industrial solvent called epichlorohydrin breaks down into (S)-3-chlorolactaldehyde, which disrupts reproductive processes in males by blocking the function of GAPD-S. Apparently, that keeps the sperm from producing the ATP needed for fertilization.

"This suggests that we can also look at infertility," Eddy says, "to see if mechanisms disrupted by mutations or environmental chemicals could be targets for mankind to disrupt. So the flip side of these studies is really contraceptive development." In other words, understanding the development of sperm could reveal weak points that might serve as specific targets for a male contraceptive. The GAPD-S enzyme in sperm could be a target for such a contraceptive. That enzyme functions only in sperm, and it is different from the enzymes used in the rest of the body. Perhaps a chemical could be designed to inhibit GAPD-S alone to make a male temporarily infertile without impacting glycolysis in any other cells. In fact, preliminary molecular-modeling studies by Rachelle Bienstock, a molecular scientist under contract at the NIEHS, indicate that differences do exist in the structures of GAPD-S and the GAPD in the rest of the body. At least some of the difference lies in the pocket on the enzyme where the sugar is held during glycolysis. Such structural differences might make just the kind of target that a male contraceptive could attack.

A variety of advances on reproductive health issues for men, from identifying environmental agents that cause infertility to the development of new contraceptives, might come with more knowledge of the regulation of genes and the interactions of proteins that drive the normal development of sperm. Eddy says, "I want what I do to benefit the health of mankind, but I'm also aware that we just need to know a lot more about basic mechanisms before we can do those kinds of things." So he will continue following his lines of basic research, but with a focus on proteins that are essential for sperm development and function, and that may be targets for environmental agents.

Mike May