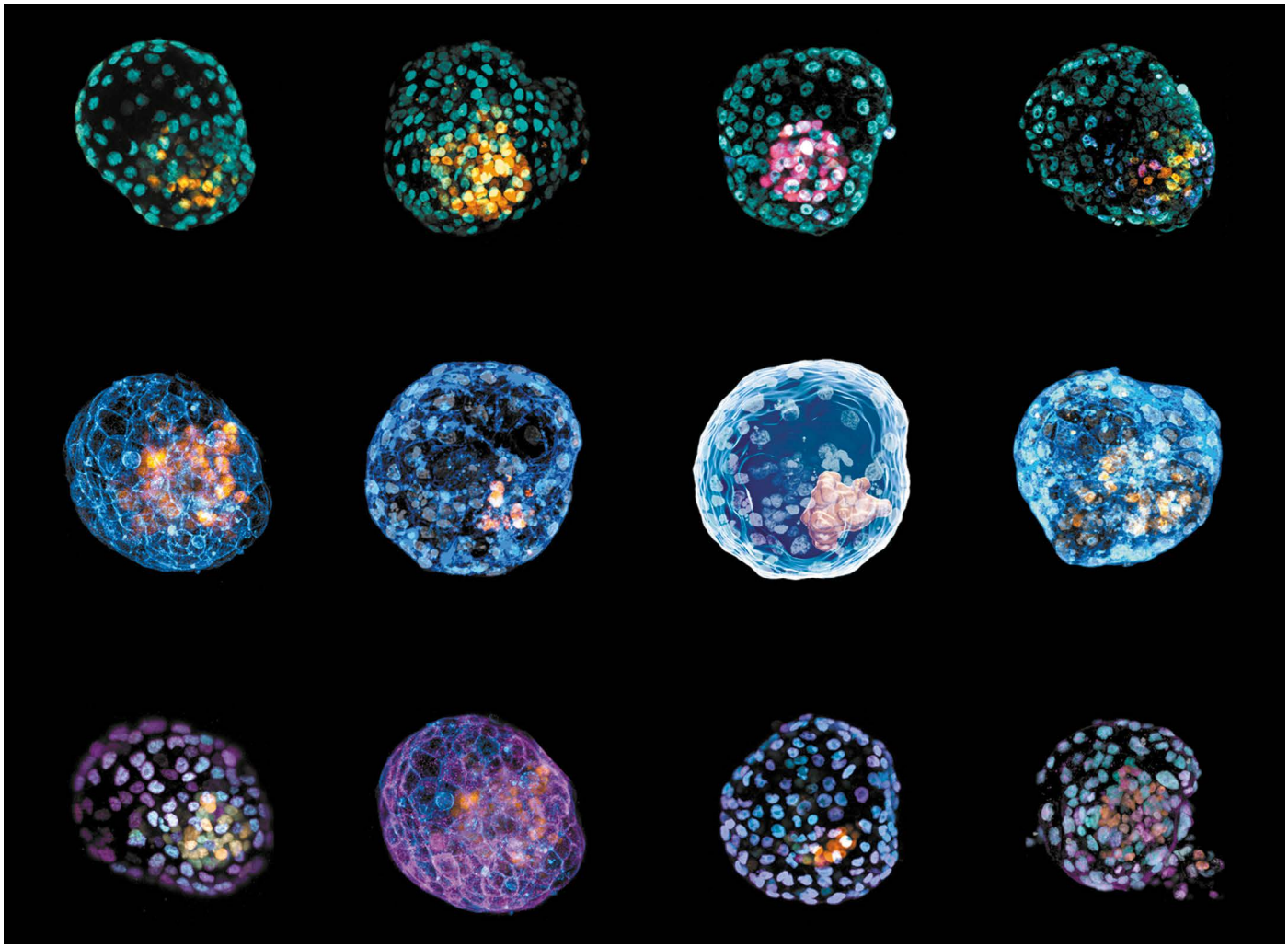

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Embryo models of the blastocyst stage of early development, reprogrammed from skin cells.

CULTURE SYSTEMS MODEL EARLY HUMAN DEVELOPMENT

Stem-cell models offer a window into human and mouse embryogenesis. **By Sandeep Ravindran**

Cells in culture typically form continuous sheets, like skin. But in 2017, bioengineer Jianping Fu realized that if he cultured human stem cells in a 3D scaffold, they would spontaneously organize into structures that looked, under a microscope, a bit like an embryo[1]. Gene-expression analyses suggested the cells were similar to those in an embryo immediately after it implants in the uterus, meaning they could serve as experimental models for a previously opaque point in early development.

"Once the human embryo implants into the maternal uterus, it becomes invisible," says Fu, at the University of Michigan in Ann Arbor. "That time frame is really a black box." But it's crucially important. This stage, about 7 to 10 days after fertilization in humans, is marked by formation of the amniotic sac and the first signs of the primitive streak. That structure marks the point at which the embryo sets up the body axes and begins to distinguish head from tail and left from right.

Researchers have long sought to observe and study these developmental stages. But working with human embryos has always been technically and ethically fraught. Animal models go only so far in mimicking humans. Natural human embryos, donated by people undergoing fertility treatment, are hard to come by. And until May this year, scientists were barred from culturing such embryos in the laboratory for longer than two weeks after fertilization. The International Society for Stem Cell Research (ISSCR) relaxed this 14-day rule in May, allowing research groups in countries where such work is legal to apply for permission to continue studies beyond 14 days.

Given these limitations, it is no surprise that researchers have sought alternatives to natural human embryos in the lab. Stem cells from species such as mice have long provided a replacement – either embryonic stem cells (taken directly from early-stage embryos) or induced pluripotent stem cells

(reprogrammed from adult cells). Now, a new wave of human embryo-like models is catching up. "If we want to understand human-specific features, we really need to look at a human system," says Naomi Moris, a developmental biologist at the Francis Crick Institute in London.

Various embryo-like models exist. They include blastoids, which mimic the stages before the embryo implants into the uterus, and gastruloids, which model formation of the body plan and organ precursors. Others include tissues that surround the embryo, such as the yolk sac and amniotic cavity (the fluid-filled sac that encloses the embryo), and some recapitulate the early steps of brain, spinal-cord and heart formation, which occurs around day 22 of human development.

Researchers are still benchmarking how similar these models are to human embryos — the greater the similarity, the greater the technical and ethical challenges. But they open up new experimental approaches. "For the first time, we are able to do genetic screens and drug screens on structures that are remarkably similar to the embryo," says Nicolas Rivron, a developmental biologist at the Institute of Molecular Biotechnology of the Austrian Academy of Sciences in Vienna. Such screens could help to identify treatments for developmental disorders or infertility. The models could also help to tease apart how organs form, potentially leading to advances in artificial transplants, for instance.

"We are using these models to study embryology from a bottom-up perspective," says Susanne van den Brink, a developmental biologist at Pompeu Fabra University in Barcelona, Spain. "It's like studying how a car works by starting at the smallest pieces and putting it together from scratch," she says.

Pre-implantation

Among the earliest developmental stages is a hollow sphere of cells called a blastocyst, which develops before implantation, when the human embryo comprises about 100 cells.

Before 2018, models of the blastocyst stage were still rudimentary. So, by combining two types of mouse stem cell in microwell arrays, Rivron and his team developed the first mouse blastoids — self-aggregating structures that resemble 3.5-day-old blastocysts[2]. Formed from embryonic stem cells and trophoblast stem cells, the blastoids revealed that signals from embryonic stem cells regulate and induce the development of trophoblast cells — the progenitor of the placenta. (This latter group of cells has a crucial role in mediating uterine implantation.) "This is a little counter-intuitive, because during later development, it's the placenta that takes care of the embryo," says Rivron. "But actually, in these early stages, it's the opposite."

Several teams translated the blastoid model to human cells by growing stem cells in a 3D inverted-pyramid culture system called AggreWell, available from Stemcell Technologies in Vancouver, Canada. They reported their findings this year. For instance, stem-cell biologist Jun Wu at the University of Texas Southwestern Medical Center in Dallas and his team built blastoids out of human embryonic stem cells by treating them with chemical factors that induce the signalling needed for blastocyst development. They found that the same method worked on human induced pluripotent stem cells generated from reprogrammed skin cells[3]. Stem-cell biologist Xiaodong Liu and colleagues at Monash University in Melbourne, Australia, likewise came up with a method to form human blastoids from human skin cells[4]. And Rivron and his team have developed a human blastoid model[5].

"That's a very nice example of how these models are starting to challenge our textbooks on embryology."

Unlike some models of human development such as Fu's, blastoids contain all three cell lineages found in natural human embryos — not just the cells that form the embryo itself but also the 'extra-embryonic' ones that lead to the yolk sac and placenta. "Human blastoids represent the first complete or integrated embryo models established from cultured stem cells," says Wu.

Using these, Wu has begun exploring the molecular signals that drive human development. By treating his blastoids with inhibitors of different versions of an enzyme called protein kinase C, for instance, he has identified which specific isoforms of the enzyme drive formation of the blastocyst cavity, the fluid-filled space at its centre[3]. "Questions concerning the molecular and cellular crosstalk between embryonic and extra-embryonic tissues during early development can only be answered with integrated models such as blastoids," he says.

Implantation and beyond

Multiple groups — including those of Magdalena Zernicka-Goetz, a developmental and stem-cell biologist at the University of Cambridge, UK, and developmental biologist Yang Yu of Peking University Third Hospital in Beijing — have used human blastocyst models to look at the stages before and immediately beyond implantation (around 7–10 days post-fertilization)[6,7].

These models use extended or expanded pluripotent stem cells, which are derived by reprogramming stem cells so that they can generate both embryonic and extra-embryonic cell lineages. Growing these cells in 3D inverted pyramid microwell plates with a combination of growth and other chemical factors allowed the cells to aggregate into blastocyst-like structures.

Both groups then extended these models into the early post-implantation stage by implanting them in a 3D scaffold called Matrigel that mimics the protein and chemical environment surrounding the cells. "[Matrigel] provides the signals to allow those stem cells to organize, and it allows us to mimic the transition that happens when an embryo implants in the body of the mother," says Zernicka-Goetz.

Fu extended his post-implantation embryo models by using bioengineered microfluidics systems made of three channels — a central gel mimicking the uterine wall, and one channel each for delivering stem cells and chemical signals in a precisely controlled manner. The system was sufficiently scalable to produce hundreds or even thousands of embryo-like structures, he and his team reported in 2019 (ref. 8). This microfluidics approach also increased efficiency and reproducibility, he says, making the models particularly compelling for translational applications such as drug and toxicity screening.

Fu used his models to demonstrate that certain aspects of human embryonic development, such as differentiation of the cells that form the innermost layer of the placenta (the amnion) from stem cells, are particularly sensitive to the mechanical rigidity and 3D nature of the matrix on which they are grown[8]. Changing the thickness of the gel matrix or growing it in 2D instead of 3D culture prevented proper amnion differentiation. "Such knowledge can only be generated, as of this moment, from *in vitro* models, because *in vivo*, it's very hard to conduct such mechanistic studies," he says.

Going past day 14

In the third week of human development (14–22 days post-fertilization), the main body pattern emerges in a process called gastrulation. So do the three 'germ layers' — the endoderm that gives rise to the gut and internal organs, the mesoderm that forms muscle and connective tissue, and the ectoderm, which creates the skin and nervous system. "These germ layers are the precursors of all organs and every cell type in the body, so it is really crucial to understand how the cellular differentiation occurs," says Berna Sozen, a developmental biologist at Yale School of Medicine in New Haven, Connecticut.

Developmental biologists Ali Brivanlou and Eric Siggia at the Rockefeller University in New York City developed the earliest gastrulation models in 2014 (ref. 9). The models "were mimicking a period of our development that we had never ever seen before, and this was one of the most beautiful things", says Brivanlou.

But those were 2D structures. Van den Brink developed the first mouse 3D models of gastrulation, called gastruloids, while working with developmental biologist Alfonso Martinez Arias at the University of Cambridge. The researchers discovered that growing small aggregates of mouse stem cells with the

correct signals, such as activators of the Wnt signalling pathway, caused them to differentiate into embryo-like structures resembling the gastrulation stage[10]. "They form a head–tail axis, the back–belly axis and the left–right axis, and they form all organ progenitors in the right location," van den Brink says.

Researchers had previously thought that, to form the head–tail axis, the embryo had to be surrounded by and receive signals from extra-embryonic tissues, which gastruloids lack, she says. "That's a very nice example of how these models are starting to challenge our textbooks on embryology."

In 2020, while working as a graduate student with Martinez Arias, Moris translated that technology to human gastruloids, finding that their patterns of gene expression roughly "followed the head-to-tail axis in these structures", she says.

By comparing gastruloid morphology to existing curated collections of human embryos, Moris was able to place her model system at about day 20–21 days post-fertilization, when structures called somites, which give rise to the vertebral column and some muscles, begin to appear[11]. "Not only were these structures recapitulating something that we knew happened in mammalian development, but it also let us kind of put a pin really where they were in the timeline of human development," she says. These models could therefore be useful in replicating these later stages of human development.

Researchers have now pushed gastruloid models even further. Brivanlou, for instance, has developed 3D models of early human-brain tissue, dubbed neuruloids[12]. By embedding gastruloids in Matrigel, van den Brink generated mouse-embryo-like models that form somites[13]. And Jesse Veenvliet, a developmental biologist at the Max Planck Institute of Molecular Cell Biology and Genetics in Dresden, Germany, generated mouse embryo-like models with somites and a neural tube — the structure that eventually forms the spinal cord[14].

"We can use the original gastruloid that doesn't have a neural tube as a blank slate and then start to add cues on top of it, so we can really start to properly dissect the minimal inputs that are needed to obtain mammalian embryo-like architecture," says Veenvliet. Researchers can even capture embryonic development as it unfolds, live on camera, he says.

Varied applications

These tools could help researchers to understand how development goes awry. Creating human embryo-like models from a person's own skin cells could allow scientists to explore the impacts of that individual's genetics on development, for instance.

Researchers such as Rivron are using organoid models of the womb lining to develop detailed implantation models, which could help to reveal causes of infertility and early pregnancy loss, and could lead to new therapies. "We can recapitulate now, in a dish, those processes that normally happen very well hidden into the womb," says Rivron.

NAOMI MORIS

Gastruloid embryo-like models are used to study formation of the body and organs.

Human embryo models could also be used to screen for drug toxicity. In a proof-of-concept study, Moris has shown that the drug thalidomide — infamous for causing birth defects in humans, but not particularly toxic in mice — had a stronger effect on human gastruloids than on mouse gastruloids, suggesting that these models could be useful for human-specific toxicological screens[15].

Human embryo-like models could even one day lead to advances in regenerative medicine, such as the ability to generate artificial organs and tissues for transplantation, says Fu. Models based on patient-derived pluripotent stem cells could grow artificial organs that the body recognizes as its own. "That's really the holy grail of regenerative medicine," says Fu.

Technical and ethical challenges

Embryo-like models can generally be made in 3–9 days using off-the-shelf consumables and protocols, and standard cell-culture skills. "You have to plate cells and then give them the right signals, but other than that, it's mostly just waiting," says van den Brink. Efficiency is often low, with only 5–20% of cell colonies developing into fully formed models, but some strategies do better. Fu's microfluidics-based models, for instance, can exceed 90% efficiency, although they require specialized bioengineering expertise.

Start with high-quality cells, advises Rivron. "Having pristine stem cells to start with is absolutely crucial." That means using cells that haven't been cultured for too long, and keeping them as healthy as possible. "Culturing them is kind of an art," he says.

Existing protocols provide a good starting point, but be prepared to fine-tune experimental conditions for optimal results. "If you're experienced in cell culture, then you can learn how to make gastruloids in about two weeks," says van den Brink.

Once they're grown, be sure to compare your models to natural embryos using both microscopy and single-cell gene-expression analysis, to ensure they are what they appear to be. "Looks can be deceiving," says Rivron. "You have to be able to understand the cells that you form and benchmark them to the cells of the embryo," he says.

Such comparisons become more difficult past day 14, because such experiments were completely banned until earlier this year, and still remain technically challenging. But that could change as techniques improve and labs apply for exceptions to the day-14 rule. "It would be very useful and would definitely allow us to validate the human gastruloid system," says van den Brink.

But ethical considerations also become more complicated past day 14. The latest ISSCR guidelines differentiate between models that contain only a part of the embryo — such as gastruloids — and those that contain all three cell lineages, such as blastoids. The latter models are subject to the same intensive review process as natural embryos before they can be studied past day 14 (ref. 16). "There's also a consensus that none of the models should be used for human reproduction purposes, and none of the models, whether they are complete or not, should be used for implantation," says Fu, who helped to draft the guidelines.

As human models of embryogenesis improve, further ethical discussions are only going to become more important. "This is moving forward now with a speed that I could have not even imagined a few years ago," says Brivanlou.

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