Modeling the early embryo

Widespread adoption of new embryo models hinges on how faithfully they mirror real embryos.

Vivien Marx

Pregnancy is a busy time: a crib is needed, diapers and baby clothes. Busy also describes the events inside the body. The earliest phases of development cannot readily be experimentally tracked or perturbed, for ethical reasons, among others.

In real life, after egg meets sperm, a zygote is formed that cleaves and then transforms into a blastocyst, a cell ball with an outer cell layer and an inner cell mass. If the blastocyst anchors itself in the uterine wall, development continues to the gastrula stage. After around nine months, if all goes well, a baby will take up residence in the assembled crib.

Embryo models to characterize the molecular and physiologic events that take place during early embryogenesis are emerging. The models are derived from various types of stem cells with differing cell medium cocktails. They are a boon to developmental biologists who seek to explore basic research questions and try to identify contributors to congenital disorders or miscarriages.

Scientists describe the models of early embryogenesis, such as the 8-cell embryo-like cell, blastoids and other blastocyst-like structures, or gastruloids, with caution. They 'resemble', 'mimic' or 'are similar to' human embryos. Labs must heed the updated guidelines of the International Society for Stem Cell Research (ISSCR)[1] related to human embryo and stem cell research. Models can fall in different categories: some are exempt from review, others are reportable, some require specialized oversight and some are prohibited.

Discussions are underway on how to assess how true these models are to the 'real thing'. For example, one can compare transcriptomes; but biological comparison is complex. Embryo models are derived from stem cells such as reprogrammed skin cells or various types of human pluripotent stem cells. What separates them from the real thing is that they have "not been derived by fertilization," says Christine Mummery, developmental biologist in the anatomy and embryology department at Leiden University Medical Center. As model developers explore, compete and squabble over how to gauge their models, they face the biological reality that a human embryo's traits are hardly an open book.

Embryo models are emerging, and one big question is how to assess how true to the 'real thing' these models are. Credit: A. Brookes

The word blastoid describes blastocyst-like structures, says Nicolas Rivron from the Institute of Molecular Biotechnology in Vienna. Words, he says, have to reflect research reality, and thus "synthetic embryos" is avoided as misleading. The '-oid' suffix indicates similarity or likeness in ancient Greek. In his view, it was his lab's mouse blastoid that triggered interest in experimenting, and competing, on ways to generate blastoids with human cells.

In 2018, Rivron, then at Hubrecht Institute for Developmental Biology and Stem Cell Research and Maastricht University, along with colleagues also at other institutions, published a mouse embryo model made with embryonic stem cells and trophoblast stem cells[2], for which they coined the term 'blastoid'. In 2021, his lab made blastoids with human cells[3].

As the model-builders nudge models along, says Mummery, decisive parameters are still emerging that say what, for instance, makes a blastocyst-like structure indistinguishable from a real blastocyst. "I would say, in general, some are a bit closer than others, or some have features that resemble blastocysts extremely strongly, but lack other features," she says. Features lacking in one model can exist in another.

The focus on validating and assessing the embryoids will guide others in their model choice. "This is the beginning of a field," says Miguel Esteban, a researcher at the Guangzhou Institutes of Biomedicine and Health of the Chinese Academy of Sciences. It's a field in its infancy that is still perfecting approaches, troubleshooting variability and discussing which markers and other assessment parameters are most useful. Results can differ across cell lines and an experimenter's experience levels. "How do you tell the difference between really identical or similar?" he asks. "It's very difficult."

What's that ball?

A human zygote undergoes a burst of activity called zygotic genome activation. The zygote

cleaves into two, then four, then into a ball of eight cells, which is when the major burst of genome activation is thought to happen, says Esteban. Details are, for the most part, unknown. For a short time, all fates are possible: this ball is totipotent and the cells can become any cell type. Esteban and colleagues generated a model of this eight-cell ball, which they call the 8-cell embryo-like cell (8CLC)[4].

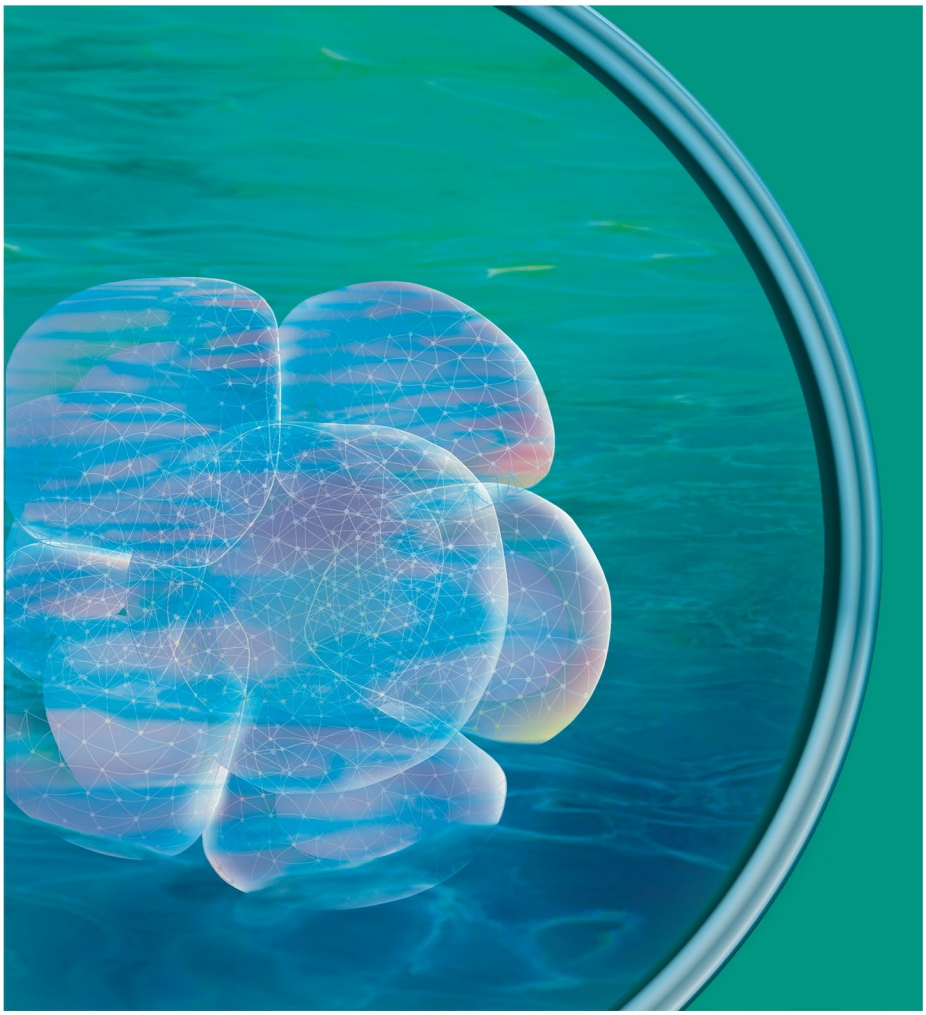
Protocols to nudge stem cells along a developmental path and shape cell fate, such as those used to make blastocyst-like structures, involve media with specific components. For the 8CLC, the team adapted one such protocol to turn back the clock on human pluripotent stem cells in vitro. As they assessed events in many cells with single-cell RNA sequencing, he says, "we started seeing that population moving day by day towards the very early beginning of everything."

This was exciting and energizing, says Wenjuan Li, a member of the Esteban lab and study co-author, and it took them right to the next step of assessing molecular events. Another co-author, Longqi Liu, who directs BGI Research's Cell Science Institute, used single-cell techniques such as full-transcript single-cell RNA sequencing and single-cell ATAC-seq (assay for transposase-accessible chromatin with sequencing) to characterize and define the transcriptomic similarity between 8CLC and the eight-cell state of a human embryo.

This 8CLC can continue development and make embryonic and extraembryonic lineages. When implanted into mice it can create teratomas, which are embryoid tumors that are considered measures of the developmental potential of pluripotent stem cells. Pluripotency means cells can take many development paths but cannot develop to placenta or umbilical cord. Teratoma staining revealed tissues derived from the three germ layers: ectoderm, mesoderm and endoderm. And single-cell RNA sequencing showed that extraembryonic lineages were present.

8CLC only mimics the human eight-cell embryo, says co-author Md. Abdul Mazid, another Esteban lab member. Indeed, says Li, in spite of the many similarities with an eight-cell embryo, "it's still an in vitro model and not generated by fertilization," she says. One difference from 'real' eight-cell embryos rests with gene regulatory networks. Two "master regulators," DPPA3 and TPRX1, emerged in their analysis, says Esteban. Both are necessary for 8CLC induction and offer insight for comparing mice and people.

DPPA3 regulates DNA methylation during development, and its mouse sequence differs greatly from the human one, says Li. TPRX1 belongs to a transcription factor family absent in the mouse. "It will be interesting to further explore how this family works in 8CLC regulation," she says. The plan is to use high-throughput screening approaches to systematically find and characterize how regulators facilitate the transition of human pluripotent stem cells into 8CLCs, says Liu.



Miguel Esteban and colleagues generated a model of the human embryo's eight-cell stage. To do so they turned back the developmental clock on human pluripotent stem cells. Seen here is a conceptual illustration. Credit: Inspired by an illustration from the Zernicka-Goetz lab, https://wellcomecollection.org/works/zjdt9yak. Illustration: W. Li, M. Esteban; M. Lu, W. Peng, H. Xiao, BGI Research Shenzhen.

These gene regulatory networks, and others, might steer developmental stages and could be implicated in disease susceptibility, says Esteban. To get "a bird's eye view of human development in vitro," he says, the team is building a database for computationally predicting development.

8CLC is a valuable model for biomedical research and for reaching a deeper understanding of what it means to be human, says Esteban. More knowledge about early development can serve studies of congenital disorders; it's potentially useful for reproductive and transplantation medicine and can shed light on species differences. Even though mice and humans are genetically similar, perhaps early development events set us apart. "This is something we are studying now," he says. If other labs want to give the model a try, "I think our protocol is not complicated," he says. "It works with multiple cell lines."

A blastocyst-like ball

With their human blastocyst model[3], the Rivron team found that it takes the combination of three inhibitory signals to generate robust, efficient, "faithful blastoids." It's the ERK, TGFβ and Hippo pathways that instruct human pluripotent stem cells to become the cell types that define a blastocyst with three founding lineages from which, later in development, three germ layers form, he says.

There is beauty, says Rivron, in the way scientists can start off with the right cells, add

the right three signals and then sit back and watch "as the events that lead to blastocyst formation unfolds in front of your eyes," he says. "It's self-organization at play!"

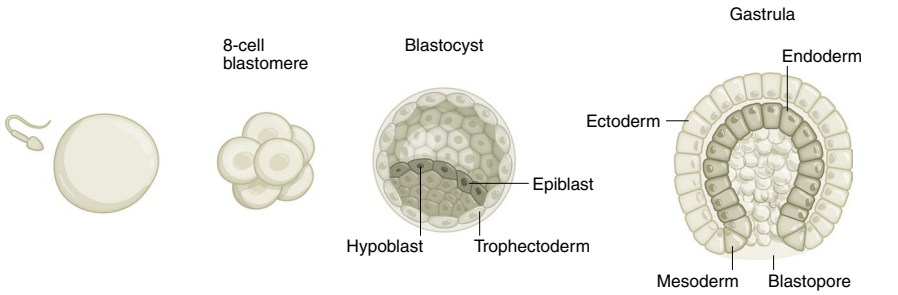
The human pluripotent stem cells (hPSCs) form trophoblast cells, which later mediate uterine implantation and form the placenta; the epiblast gives rise to the body of the embryo. In a second step, the primitive endoderm cells and the polar trophoblasts emerge, which are the 'sticky' cells that anchor the embryo in the uterine wall. After fertilization and before implantation into the uterus, human blastocyst development takes 7 days. At around day four, a morula develops, says Rivron, that's the pre-blastocyst stage. "From morula to full-blown blastocyst-stage, it takes 3.5 to 4 days." It takes hPSCs 4 days to generate blastoids. To him, "hPSCs unleash their inherent potential in an efficient, faithful and timely manner."

It was "incredibly thrilling," says Rivron, when the team saw in a dish that the blastoids attach to hormonally activated endometrial cells. This showed that the hPSCs recapitulate the steps of blastocyst development. Following this "whoa moment," he says, were doubts, skepticism and the dread of publishing something incorrect, which sent them into several months of validation. For independent benchmarking, they reached out to Laurent David at the University of Nantes and team. They wanted to be sure blastoids were forming the right cells in the right sequence and at the right pace. This work, he says, builds on research by "pioneers not to be forgotten" over the last decade who showed that pluripotent stem cells can form trophoblast cells.

"I still remember the moment my two postdocs who are co-first authors on the paper, Leqian Yu and Yulei Wei, came to my office and told me they could generate human blastocyst-like structures on the first trial when using naive human ESCs as the starting cells," says Jun Wu, a researcher at University of Texas Southwestern Medical Center.

This successful moment came after the lab had, for an extended period, been working to generate a human blastocyst model. The key, says Wu, is the starting pluripotency of the hPSCs[5]. They tested different types of hPSCs, such as human embryonic PSCs, intermediate hPSCs, different types of naive human embryonic stem cells and others too, and these cell cultures had differing pluripotency states.

The team found that only hPSCs grown in two naive conditions with certain media — 5i/L/A and PXGL, which contain specific kinase inhibitors and growth factors — can robustly give rise to blastocyst-like structures. They recommend using stable, naive hPSCs that have been passaged fewer than 20 times.



After egg meets sperm, a zygote is formed. It cleaves into a blastomere; further transformations lead to a blastocyst. If the blastocyst anchors itself in the uterine wall, development continues to the gastrula stage. Credit: Thomas Phillips, Springer Nature

"We were all surprised by the plasticity of naive hPSCs toward both embryonic and extraembryonic lineages, which is the key for generating blastocyst-like structures," says Wu.

In their paper, the team noted that some cells failed to match the cell state of cells in human blastocysts. The choice of single-cell sequencing platform contributed to this noise, says Wu. For their blastoids, they had used the 10X Genomics platform while the benchmarking data were from human blastocysts characterized with Smart-seq2.

Since publication, the team has been improving the efficiency and fidelity of their blastoid model, says Wu. Their latest protocol can reach 90–100% efficiency, a result other labs have reproduced, he says. Beyond protocol improvement, they have compared their data to blastocyst data obtained with a 10X platform, and "our blastoids match very well with human blastocysts at the single-cell transcriptomic level," he says.

To evaluate an in vitro embryo model, researchers can use single-cell transcriptomic analysis, but "I don't think it's a must when other labs are planning to use these models," says Wu. To validate models they generate before they use them, researchers should do some molecular and functional lineage analysis. Embryo models and embryos differ in many ways, "and we are still learning these differences," he says.

In his view, none of the blastocyst models or blastoids "faithfully resemble human blastocysts." "All models are wrong, but some are useful," he says, riffing on a quote attributed to statistician George Box. In Wu's view, his team's blastoid model is a useful surrogate for human blastocysts to enable study of early human development and implantation.

On being blastocyst-like

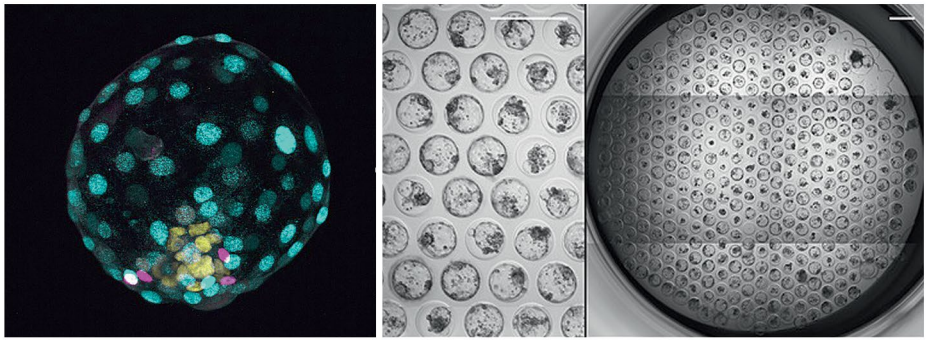
A team at University of Exeter along with colleagues at Wellcome-MRC Cambridge Stem Cell Institute, University of Cambridge and University of Tokyo have, starting with human pluripotent cells, differentiated them into a "blastocyst-like structure" with the "three founding lineages of the blastocyst."[6]

To launch trophectoderm differentiation involved inhibiting two signaling pathways — ERK and NODAL — using two small molecules; then they modulated treatment with these molecules to generate blastocyst-like structures. They performed single-cell transcriptomic analysis with Smart-seq2 to compare expression to existing human embryonic single-cell RNA sequencing data and found the data clustered into "three unambiguous lineages." Their transcriptomic data matched those of human embryos with "high transcriptome fidelity," and there were few or no unassigned cells. The time scale of morphogenesis was similar to the time course of a human blastocyst, which is three to four days.

The team used immunofluorescence-based staining to check expression of trophectoderm- and epiblast-specific markers, says Ge Guo from the University of Exeter. They stained for a hypoblast marker. "What is important is that the immunostaining is appropriately restricted to the expected locations for each lineage," says Guo. Furthermore, in the case of the trophectoderm marker GATA3, all outer cells show expression, as is to be expected in an embryo.

There was a "high degree of fidelity" in the segregation of developmental lineages when comparing blastoids and human blastocysts. "A critical point is that almost all cells in the blastoids show high similarity to cells in the human blastocyst," he says. This is in contrast, Guo says, to results presented by some other labs. The protocol shows differences in efficiency and timing between stem cell lines. "The key point is that the protocol is effective with all the lines tested," says Guo.

Says Guo, the starting point for a typical human embryo, the fertilized egg, is unlike the starting point for blastoids, which begin



Forming faithful models of human blastocysts is crucial because a lack of fidelity in the developmental processes and cellular composition will reduce the power of the model to reveal mechanisms of development and diseases, says Nicolas Rivron. Seen here are micrographs of blastoids derived from human stem cells. Credit: Rivron lab, IMBA; adapted with permission from ref. [3].

from a group of stem cells already at the epiblast stage. Among the dissimilarities to a human blastocyst is the variable number of hypoblast cells across blastoids.

Published data indicate that hypoblast cell numbers differ in human blastocysts. Says Guo, it is unknown to what degree this hypoblast cell number variability reflects natural human embryo development. This would be yet another difference between human and mouse embryos but may be connected to culture conditions. One way to resolve this issue is to generate large numbers of blastocyst-like structures to determine the true variability and screen for conditions that may regulate this variability.

"Indeed, the hypoblasts can have different numbers of cells," says Mummery. "There is natural variability," as the widespread practice of pre-implantation genetic diagnosis has shown. Routinely, biopsies are taken from embryos at the eight-cell stage. An embryo "can miss one-eighth of its cells, no problem," she says. The cells are replaced and development continues.

During development — and "it's all to do with HOX genes and things like that" — embryos "get a feeling of space," she says. Given their sense of neighboring cells, development waits until neighbors are assembled before the next stage unfolds. Perhaps hypoblasts of differing sizes have different developmental clocks. "There's all these different variables," she says. Development is flexible; various events can occur and it continues along.

A need for benchmarks

There is a need for parameters and quantitative readouts to assess the faithfulness to the real thing, and it's an aspect "a bit missing in the literature," says Mummery. Not only must labs quantitatively assess their models, they need to inquire "to what extent are they similar or the same as real values you measure in a real tissue," she says. In her experience "just by the very nature of what you can measure in vitro versus in vivo, nine times out of ten, they don't match."

Parameters can indicate similarity to a human blastocyst, yet with some features "we don't even know what they are in humans," says Mummery. Modelers can only say the structure they've made "is as similar as possible in these features to a real blastocyst as far as known."

Says Rivron, in 2021, six labs published ways to generate blastoids, with varied results. He points to an analysis of the different protocols by Ronghui Li at Salk Institute for Biological Studies and Altos Labs and colleagues[7].

When researchers assess embryo models and compare protocols, he recommends looking at general efficiency, which informs as to whether cells are "capable and properly stimulated." A few carefully curated molecules suffice to stimulate human pluripotent stem cells. "Using too many molecules will confuse them and lead to the formation of abnormal or differentiated cell types reflecting later embryonic stages," he says.

Evaluation should include assessing how long it takes structures to form. A human blastocyst typically begins to form within four days. If it takes longer for a morphologically good structure to form, the hPSCs can form abnormal or more differentiated cell types. Morphogenesis must also, says Rivron, be "tightly and timely coupled with the specification of cell lineages."

Another, albeit more complicated, evaluation approach is to assess the blastoid's transcriptome. That's expensive and time-consuming, with data that are challenging to analyze, but "it becomes a simple standard," says Rivron, when researchers use a reliable reference map of the early human embryo onto which scientists can project their data. An international consortium has built a resource that includes data from in vitro cultured human blastocysts and an in vitro gastrulation-stage human embryo specimen. The consortium team used the resource to assess cell stages in four out of six published blastoid protocols[8]. Two protocols[2,6] led to cells that are similar to those of the human blastocyst stage and two other protocols formed mostly abnormal or more differentiated cells.

Two approaches that were not analyzed use extended potential stem cells as a starting culture. Such cells are a "difficult choice," says Rivron, because these hPSCs reflect a post-implantation stage. The analysis establishes basic criteria for evaluating results; they include morphometry, cell numbers, timely and sequential specification and final cell states. Key blastoid features for the six protocols can be found in the paper by Li and colleagues[7].

As Rivron says, a meta-analysis[8] highlights that inadequate initial parameters might lead cells to, for example, form a cavity as a blastocyst does, but generate structure that can have abnormal cells or ones in other developmental stages. For instance, many epithelial tissues can form a cavity, as do organoids, but "cavitation alone is not sufficient to assert the formation of a trophectoderm-like tissue."

For blastoids, he says, the trophectoderm-like tissue is a thin monolayer of cells that encapsulate a cavity. The cavity formed later in development by the epiblast and amnion is thicker and has columnar epiblast cells and thinner amnion cells.

"Forming faithful models is crucial as a lack of fidelity in the developmental processes and cellular composition will reduce or abrogate the model's predictive power to reveal mechanisms of development and diseases," says Rivron. Beyond these basic features, it also matters that blastoids attach only to activated endometrial cells, and via the polar trophectoderm analogs, which correspond to "sticky cells of the blastocyst." This increases confidence in the model's functionality and opens avenues for mechanistic investigations of human embryo implantation and early development.

Given the robust, efficient protocols for forming blastoids, he says, some simple and regular quality controls are suited to assessing the initial state of the cells and the reagents. Scientists need to pay careful attention to features of the resulting blastoids: morphology, immunostaining for the three lineages, and staining to assure absence of markers of differentiated cells that reflect later embryonic stages and abnormal cells. That, in his view, "is sufficient to maintain the high standards necessary to perform mechanistic studies." It takes a few months

Yes to standards

"We must establish standards about the minimal features depicting a blastoid," says Nicolas Rivron. He and his colleagues suggest that blastoid- or blastocyst-like structures should conform to these basic standards: they form the right, blastocyst-like cells in the right sequence — trophoblast and epiblast cells first, polar trophoblast and primitive endoderm second — and all of this should unfold at the right pace. Initial cell state and culture conditions must allow specification, morphogenesis and patterning to be tightly coupled such that blastocyst-like cells are the result.

Standards will help the embryo modeling field advance, says Mummery, but biology does not make this a straightforward task. As a comparison, she says, scientists can measure the barrier function of a vein's or artery's vascular wall. That is easier to do in vitro than in a living animal. Secreted products in a person's bloodstream at certain levels can indicate a myocardial infarction. A 'stressed' model that mimics a myocardial infarction might also generate those same markers. "Then the question is, what about the level of those markers?" How best to compare the quantitative measurement to the in vivo one? "Is it 10 or 100 times lower, or yes, bigger?" When modeling the heart, for example, assessment must include the size of the organ relative to the amount of blood in the bloodstream. And there is also a heart's electrical behavior to take into account. Such considerations for heart models apply to embryo models. Labs are busily working out, she says, which existing data can serve as model benchmarks, and establishing standards is a task the ISSCR is taking on.

of diligent monitoring to get a feeling for the assay and to determine whether the initial hPSCs culture and resultant blastoids are of high enough quality to conduct scientific experiments.

Further on, gastruloids

The question of what a stem-cell-derived cell needs to be "is a tricky, tricky thing," says Mummery. "What it has to show may only be what's necessary for your particular assay." For a liver cell derived from stem cells, that might be expression of P450 enzyme, a toxicology standard. But in the context of a potential transplant, other parameters are needed to know whether the cell fulfills all the duties it should.

With blastoids, the "ultimate test" is to see if a blastoid or blastocyst-like structure can develop into an embryo, says Mummery. "If it can, then by definition, it is a blastocyst," she says. But for humans, according to ISSCR guidelines, such experiments are prohibited. It's only permissible to implant a mouse blastoid into a female mouse and see whether it develops into a healthy newborn and then an adult mouse.

A mouse blastoid might look like a human blastocyst, but that does not mean it corresponds to one, says Mummery. There is, as of yet, no full characterization of the human blastocyst right after implantation. The ISSCR guidelines categorize blastoids in a higher ethical risk group than gastruloids. With blastoids, one can potentially add in the trophectoderm, which would model all of a human blastocyst's cellular components, she says. "So you're very close, actually, to having an embryo."

What also matters is the order in which a lineage arises and where it is spatially situated. "If you're looking at regions that have to signal to each other," she says, and one of those partners is absent at a particular time, development might not proceed.

A gastruloid, which is a gastrula model, looks more like an embryo but it lacks trophectoderm and other extraembryonic tissues. One cannot simply add trophectoderm to a gastruloid. "It wouldn't be at the right stage," she says and it's too late in development to be integrated. Extraembryonic tissues have a developmental trajectory and interact with the embryo to develop. Thus, gastruloids may appear more similar to an embryo but present fewer ethical challenges, she says.

On the other hand, gastruloids can make germ cells. Once a lab does that, the experiment moves into a different, "slightly more ethically sensitive area," she says. Making sperm or eggs in the lab is ethically unproblematic, but using these sperm or eggs for fertilization falls in a different category. Such work has been done in mice and in rats, she says, "but that's not allowed for humans."

"I'm a very strong believer in gastruloid models," says Mummery. "Blastoids, I'm not entirely sure; it's very, very early." In a pregnancy, if something is amiss with a blastocyst, it can lead to an early miscarriage. Aspects of congenital defects such as microcephaly or altered sidedness of the heart might be more readily studied in gastruloids.

In mouse gastruloids and perhaps soon in human gastruloids, one can study left–right asymmetry, an aspect that can go awry in heart development. Models can be used to explore why a heart develops missing a ventricle. Some individual causative genes have been identified, but researchers are looking for the many genes or predisposing factors, such as single nucleotide polymorphisms. Gastruloids can also help to study somite formation, which is part of muscle and limb development. Early brain development, too, can be studied with gastruloids. What she finds exciting is having the opportunity to study such events without using gastrula-stage embryos.

Mummery was ISSCR president when the 2021 updated guidelines were discussed, but she was not directly involved in developing them. It was "a huge amount of deliberation," she says. The shorthand sometimes used, falsely, she says, is that ISSCR has dropped the so-called 14-day rule for culturing embryos. What is true is that ISSCR proposes to relax the 14-day rule in certain well-described cases.

There are different categories of required oversight for experiments with stem-cell derived models of human embryos. There are categories exempt from oversight, such as non-integrated stem-cell-based embryo models, of which gastruloids are one type. They lack the lineages of a human gastrula. Specialized scientific and ethics review is required for integrated embryo models, which have all the cell lineages an embryo needs to develop. Some approaches are prohibited, such as gestating human stem-cell-based embryo models.

Once labs know, for example, the characteristics of an 18-day human embryo, this might offer insight, and "we can do a lot more interpretation of what these gastruloid models mean." For all embryo models, an important question labs will likely need to ask themselves is: "is my model fit for purpose?" ❐

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