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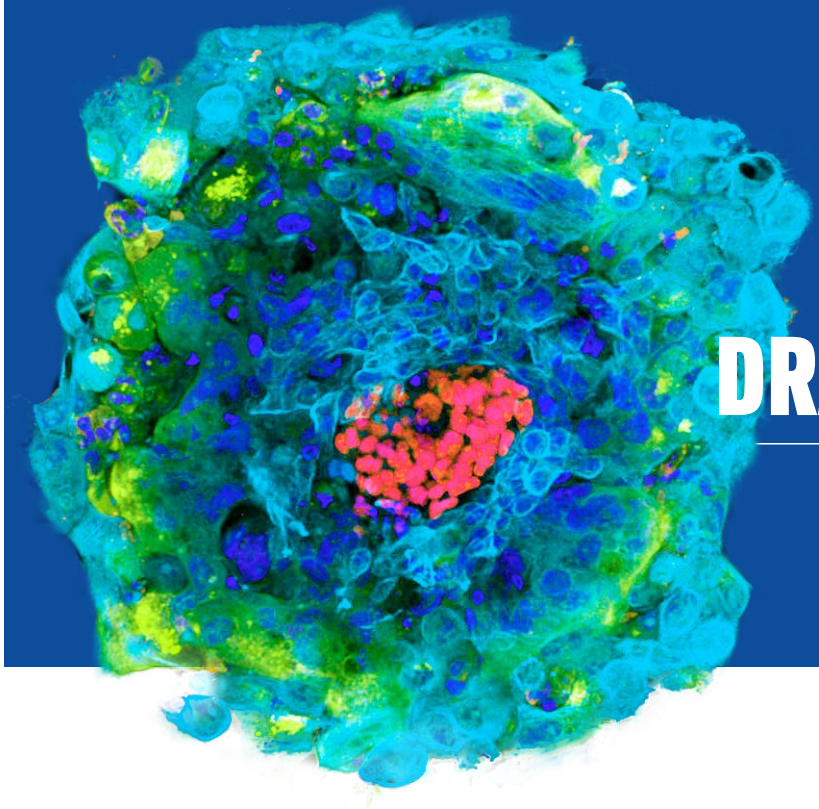

DRAWING THE LINE

ETHICAL, POLICY, AND SCIENTIFIC PERSPECTIVES ON U.S. EMBRYO RESEARCH

HUMAN EMBRYO RESEARCH: WHAT DO WE KNOW AND HOW DO WE KNOW IT?

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"Human Embryo Research: What Do We Know and How Do We Know It?"

Introduction

How do humans go from one cell, too small to see with the naked eye, to the more than 30 trillion cells that make up a human body (Bianconi et al. 2013; Sender, Fuchs, and Milo 2016)? From the earliest step, the fusion of a single egg and sperm, through the first eight weeks after fertilization, human embryo development requires many complex processes that we are only beginning to understand. Our understanding is largely limited because our knowledge of human development comes from a few human embryo specimens. With this limited number of embryos, scientists cannot examine human embryo development with the same reproducibility or detail as they do model organisms, such as frogs and mice.

From animal models, scientists have discovered that embryo development is a dynamic process. There are complex cell movements and repeated interactions between cells that are required for normal development. Experiments increasingly require examination of live embryos to understand these complex interactions. Unfortunately, we have limited understanding of how these processes occur in the human embryo. This problem is compounded by the unique geometry of the early human embryo, which makes direct comparison between animal models and humans challenging.

The embryonic period from conception to eight weeks of development is a period when the basic plan of embryo organization is laid down.

Investigating human development teaches us about ourselves and improves our understanding of the genetic and environmental causes of health conditions like infertility and congenital diseases. The embryonic period from conception to eight weeks of development is a period when the basic plan of embryo organization is laid down. The foundation for future organ development is established and elaborated. Many congenital birth defects and later-manifesting diseases have their roots in events that occur during embryogenesis (the first eight weeks of development). Furthermore, it is estimated that a third of human conceptions result in spontaneous abortion (Schoenwolf et al. 2015). About half of these have clear chromosomal abnormalities, but the causes are mostly unknown for the other half (Nagaoka, Hassold, and Hunt 2012; Hassold et al. 1996). Thus, understanding embryogenesis will have long-reaching implications for our comprehension of the genetic and environmental causes of human disease.

The goal of this paper is to outline scientists' current knowledge of human embryo development. We review the use of model organisms to understand vertebrate development and the methods that developmental biologists use in these models. Investigation with these model organisms seeks to improve our understanding of human development by capitalizing on the similarities among animals. However, these techniques can be limited by the differences between organisms. To highlight the complexity of embryo development, we provide an overview of the development of the central nervous system (CNS), especially brain and spinal cord development. We also describe features of

embryos at different stages during embryogenesis. In the end, we hope to highlight what we know, how we know it, and what is still unknown about early human embryo development during the first eight weeks after fertilization.

Knowledge Obtainable from Model Organisms

A 70 kg (~150 lb) adult human male has between 30 trillion to 40 trillion cells (Bianconi et al. 2013; Sender, Fuchs, and Milo 2016). In the early embryo, cells replicate and become different from each other by producing different gene products such as proteins that move to specific parts of the embryo and take on specific shapes. This differentiation process results in the production of hundreds of identified cell types that are distinct in their shapes, locations, and functions. Making 40 trillion cells from a single cell would only require 45 ideal cell divisions if each was symmetrical ($2^{45} = 35 \times 10^{12}$), but biology is a little more complicated than that. Some cells give rise to many cells and cell types, and others to only a few. Add to this the constant requirement to turn over tissues, such as skin, gut lining, and blood, and things get complicated fast.

How the human body arrives at this complex, deeply integrated sum of its parts is the question that drives developmental biologists. Most developmental biologists focus on organisms other than humans for ethical, technical, and financial reasons. Ethically, we are compelled to limit the number of human embryos that are used in research. There are several guidelines that describe approval processes to oversee this type of work, including guidelines from the National Academies of Science, Engineering, and Medicine (NASEM) and the International Society for Stem Cell Research (ISSCR) (NASEM 2008; ISSCR 2016).

Most of our knowledge about human embryo development is extrapolated from work on model organisms.

Technical limitations to human embryo research involve the relative scarcity of human embryos available for research. Historically, our ability to analyze embryos has been limited to specimens provided by centralized embryonic and fetal research centers, but these are mostly fixed or frozen tissues for molecular analysis (Gerrelli et al. 2015). Thus, these samples are just snapshots of an embryo at one moment in time during a dynamic process. Being able to watch an animal embryo change and grow in real time has transformed our understanding of embryo development in model organisms. To truly understand human embryonic development, such experiments need to be repeated, ultimately requiring millions of embryos to test the different processes that occur during development (Hoffman et al. 2003); but *in vitro* fertilization (IVF) clinics could supply, at most, only several thousand human embryos for research. Furthermore, the use of federal money for research that results in the destruction of a human embryo is prohibited in the United States (see the paper on U.S. policy for more details). These regulatory restrictions are compounded by local laws that may further restrict research.

Box 1. Glossary of Embryology Terms

Amnion – An extraembryonic structure formed prior to gastrulation in humans and during gastrulation in mice.

Blastocoel – A cavity that forms in many blastula stage embryos.

Blastocyst – A blastula stage embryo that has an expanded, central, fluid-filled space or blastocoel.

Blastodisc/blastoderm – The region of the embryo that will give rise to the embryo, distinct from extraembryonic tissues.

Blastula – A rapidly dividing embryo before obvious differentiation within the embryo proper.

Bone morphogenetic proteins (BMP) – A subfamily of secreted signaling proteins that cells use to communicate with each other.

Caudal – The tail or posterior end of the embryo.

Commitment – Restriction of developmental potential. A committed cell cannot take on a different fate when transplanted into a new location.

Differentiation – The act of cells becoming different from other cells. Typically requires that cells express proteins or mRNAs (messenger RNA) indicative of their ultimate fate.

Ectoderm – The outside layer of the embryo that gives rise to organs like the outer layer of the skin and the central nervous system.

Endoderm – The internal layer of the embryo that gives rise to the lining of the gut, lungs, and organs like the liver and pancreas.

Epiblast – The sheet of cells that will give rise to the embryo proper.

Extraembryonic tissues – Cells that support embryo development such as those that form the trophoblast, yolk sac, and placenta. They may produce signals that pattern the developing embryo but will not contribute to the embryo itself.

Gastrula – Embryo in the process of rearranging itself to create the three cell layers (known as germ layers): ectoderm, endoderm, and mesoderm. The period of time this is occurring is called gastrulation.

Hypoblast – A sheet of cells that will give rise to extraembryonic tissues that form under the epiblast.

Fate map (lineage map) – A map of the ultimate fates of cells in an earlier stage embryo. An accurate map requires that development of the embryo be reproducible despite the fact that cells will undergo dramatic rearrangements during development.

Mesoderm – The middle layer of an embryo that gives rise to organs in between the ectoderm and mesoderm, such as muscles, kidneys, and most bones and cartilages.

Morphogenesis – The sum of cell movements, cell divisions, and cell shape changes that rearrange parts of the embryo in response to coordinated cues so it progressively takes its mature shape.

Morula – A compacted early stage mammalian embryo prior to the blastula stage.

Neural crest – Cells at the junction between the forming epidermis and neural plate. These cells migrate away from their site of origin and make many different cell types, including the bones of the face, pigment cells in the skin, and the nervous system of the gut.

Placodes – Groups of cells at the junction between the forming epidermis and neural plate. These cells give rise to different structures including the sensory cells in the nose, the lens of the eye, and parts of the ear.

Rostral – The nose or anterior end of the embryo.

Specification – The process of receiving patterning signals and expressing genes that indicate cells have begun to take on a new fate. Cells that are specified but not committed can take on new fates when transplanted to a different part of the embryo.

Trophoblast – Extraembryonic cells that form as the external layer of the blastula stage embryo.

Wild Type Gene – A normal, fully functional gene (unmutated).

Zygote – A newly fertilized embryo that consists of one cell.

Given the constraints imposed on working with human embryos, most of our knowledge about human embryo development is extrapolated from work on model organisms. Model organisms are representative species that scientists use as a proxy for other organisms, including humans (Table 1). Each model can teach us a great deal about similar organisms, but no model is a perfect replica of a human or any other organism. Some offer specific technical advantages; for instance, some models are easier to manipulate or observe, while others have vast genetic resources available. With a careful analysis and thoughtful interpretation, even distantly related organisms can teach us about the human embryo. One only has to look in any modern text on human development to see that it is full of pictures of model organism embryos that provide the basis of our understanding for what is happening in human embryo development.

Animal Models of Human Development

When addressing specific questions of human development, investigators must use great care in choosing questions that are appropriate for the model organism. As indicated earlier, model organisms are important developmental biology tools for scientists. They allow scientists to examine and manipulate embryos at many different stages in order to gain a better understanding of development. Experimental developmental biologists test hypotheses by observing embryos from model organisms and testing the consequences of changing the activity of specific genes, rearranging parts of the embryo, or adding environmental challenges. As described in the next section, these manipulations allow scientists to determine the functions of specific genes in specific processes, identify how cells communicate with each other, and understand how cells change their behavior in response to their environment. The specific methods used vary depending on the model and the specific process being examined. However, these studies require large numbers of embryos due to technical challenges and to ensure accurate interpretations when results vary.

Scientists rely heavily on nonprimate vertebrate model organisms—both nonmammalian and mammalian—to help elucidate human development. Nonmammalian vertebrate model organisms include zebrafish, frogs, and chickens. All of these models have common features. Embryos can be obtained in large numbers. With the exception of chickens, the adult animals are easy to keep in the lab and breed to obtain embryos (fertilized chicken eggs are easy to obtain and transport from breeders). Furthermore, embryo development is very regular and predictable. The nonmammalian models also develop outside of their mothers, which provides a number of advantages to developmental biologists. For instance, scientists can obtain large numbers of embryos. Similarly, they can be viewed easily over long periods of time, allowing detailed observation of living embryos and mapping of cell fates. These embryos are also amenable to genetic manipulations and drug treatments since they are accessible to microinjection and surgeries due to their external development. Each of these models has taught us a great deal about the species' own development, the development of closely related species, and what probably happens during different stages of human embryo development. In fact, many human genes have names that are derived from the related genes in fruit flies. Unfortunately, these nonmammalian models have considerable differences in embryo shape, size, composition, and developmental timing that prevent our understanding of precise aspects of human embryo development (Figure 1).

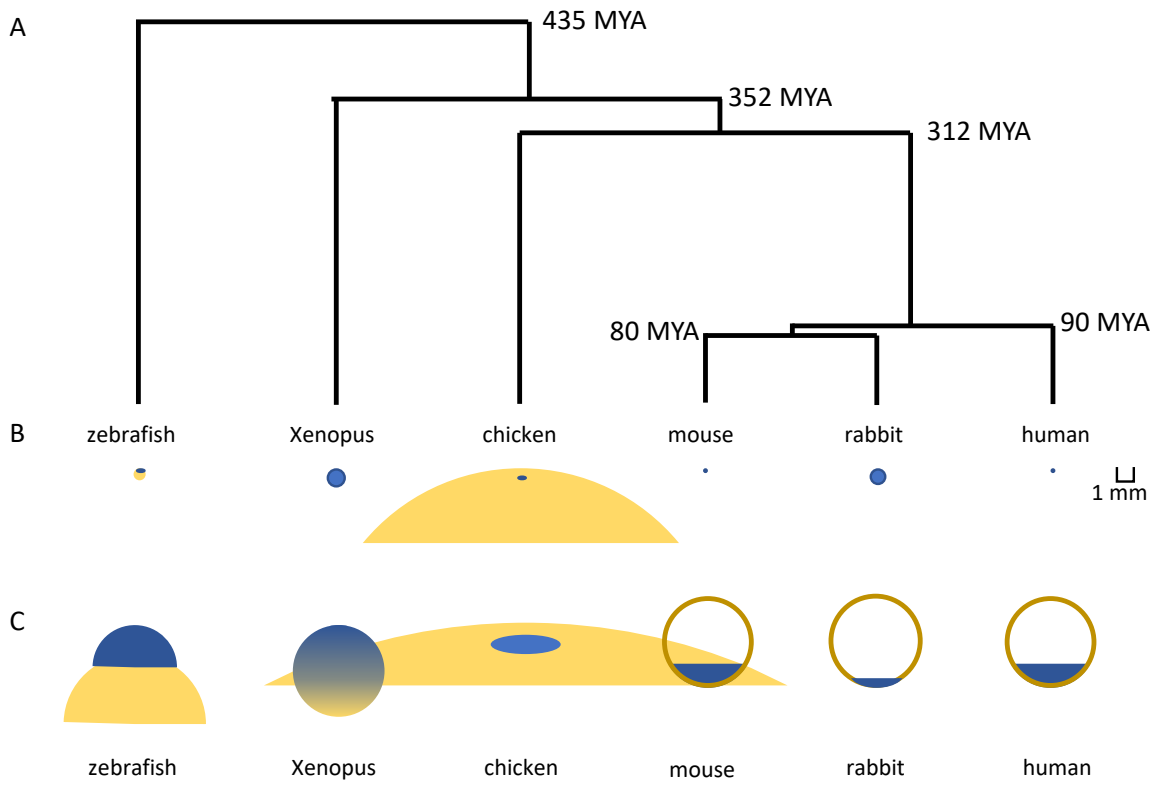
Table 1. Nonhuman Embryos Each Have Experimental Advantages and Disadvantages.

Organism	Scientific Name	Last Common Ancestor with Humans	Robust Genetic Tools	Expensive to Maintain	Easy to Culture and Image Live Embryos	Easy to Create and Keep Mutant Lines	Comments
Fruit fly	*Drosophila melanogaster*	797 MYA	Yes	No	Yes	Yes	Long-established and robust
Worm	*Caenorbaditis elegans*	797 MYA	Yes	No	Yes	Yes	Long-established and robust
Zebrafish	*Danio rerio*	435 MYA	Yes	No	Yes	Yes	Long-established and robust
Frogs	*Xenopus laevis, Xenopus tropicalis*	352 MYA	Yes	No	Yes	Yes (*X. tropicalis)*	Long-established and robust
Chicken	*Gallus gallus*	312 MYA	Yes	No (eggs & embryo)	Yes	No	Long-established and robust
Mouse	*Mus musculus*	90 MYA	Yes	Modest	Modest	Yes	Long-established and robust
Rabbit	*Oryctolagus cuniculus*	90 MYA	No	Yes	Modest	No	Long-established biomedical model, including reproductive biology. Emerging genetic model with new mutation methods but cost is much higher than mice
Common marmoset	*Callithrix jacchus*	43 MYA	No	Yes	No	No	Emerging model with new mutation methods but cost is much higher than mice
Rhesus macaque	*Macaca mulatta*	29 MYA	No	Yes	No	No	Long-established biomedical model, including pioneering work on embryonic stem cell derivation. Very expensive; most work restricted to primate research centers
Chimpanzee	*Pan troglodytes*	6.6 MYA	No	Yes	No	No	**Currently retiring in U.S. research**

Note*:* MYA – million years ago

Figure 1. Vertebrate model organisms have diverse sizes and arrangements of the blastula stage embryo.

(A) A phylogenetic tree showing the relationships and estimated last time of divergence for diverse vertebrate model organisms. **(B)** Blastula stage embryos of these groups have different sizes, as depicted in this relative scale schematic. **(C)** An unscaled schematic illustrates the diverse distribution of extraembryonic tissues (yellow) relative to the embryonic blastoderm (blue) at blastula stages.



Nonmammalian embryos have significantly different organizations from human embryos. External development requires the investment of a large amount of yolk into the egg. This is most obvious in the chicken embryo, where the blastoderm, or early embryo, sits on top of a yolk cell that is several centimeters across (Figure 1). Differences in the shape of the embryo reflect differences in the sources of signals that pattern it. For example, the large yolk cells of fish and frogs are sources of signals that tell the cells next to them that they will become endoderm and ultimately make the gut, liver, and pancreas among other organs. But these yolky cells do not exist in mammalian embryos, so a different source of similar signals evolved to serve this function (Fukuda and Kikuchi 2005).

Nonhuman Mammalian Animal Models

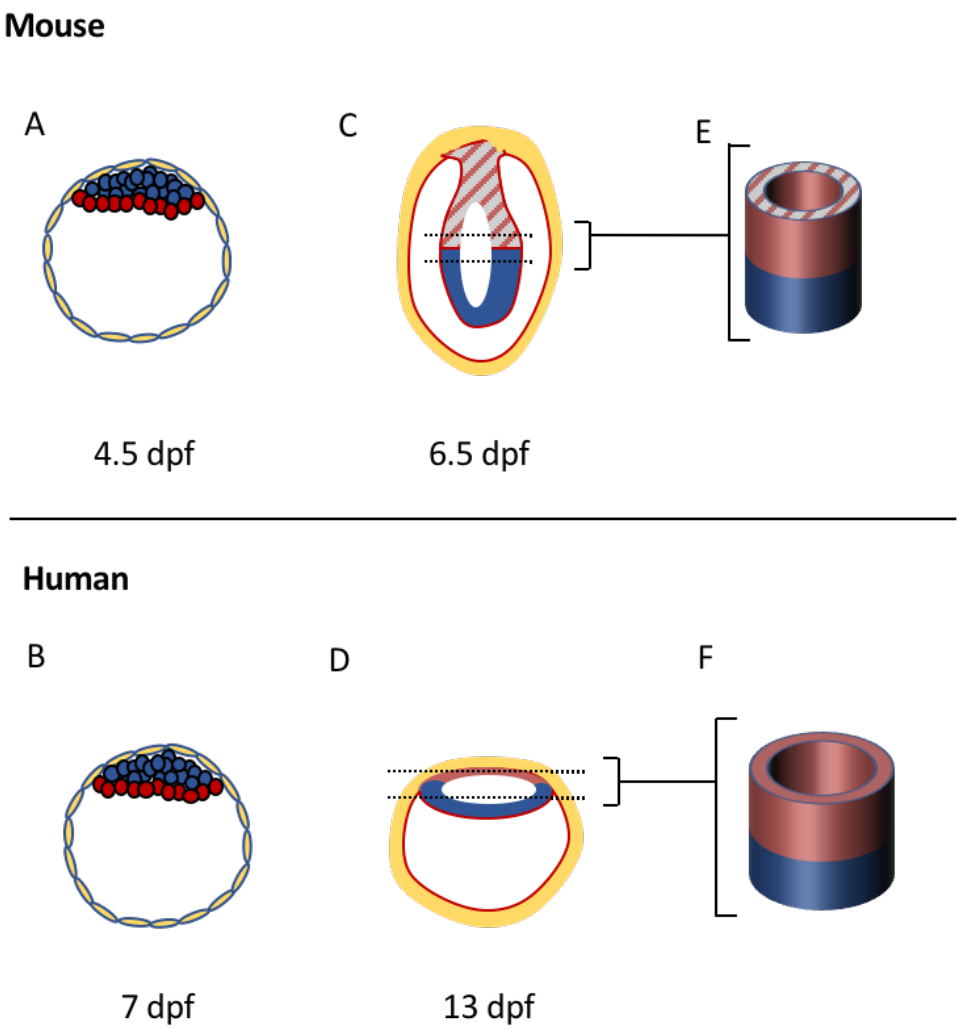
Mammalian organisms are also used as models of early human development. The mouse has been by far the most popular model for developmental genetics since genome engineering and genetic manipulations in mouse embryonic stem cells (ESCs) were developed in the 1980s. Mice are robust in the lab, as they breed easily and have simple requirements to keep them happy and healthy. Through careful experimentation, methods for *in vitro* culture have been developed that allow for the creation of transgenic embryos (embryos with synthetic or artificially mutated genes) and genome manipulation (Brinster and Palmiter 1984; Evans and Kaufman 1981; Mansour, Thomas, and Capecchi 1988; Martin 1981). ESC gene targeting allowed for a large number of genes to be mutated and manipulated to understand their roles in the development and physiology of the mouse. These technologies have improved over the years, and now the genome of the mouse can be engineered with more detail than in any other animal (van der Weyden et al. 2011).

A major disadvantage of using mice as a proxy for understanding human embryo development is that rodents have a unique morphology around the time of gastrulation, with the embryo rudiment forming a cup shape within the extraembryonic tissues, rather than the disc shape of the human embryo (Figure 2). This developmental difference moves the source of signaling centers relative to the developing embryo and may cause differences in the roles of specific signaling molecules in patterning the embryo at these stages. One prominent example is the differences in primordial germ cell origin between humans and mice. Mouse primordial germ cells are induced on the edge of the epiblast (Lawson et al. 1999; Lawson, Meneses, and Pedersen 1991; Saitou, Barton, and Surani 2002), while human primordial germ cells are induced in the amnion (K. Sasaki et al. 2016; Kobayashi and Surani 2018), an extraembryonic tissue that forms prior to gastrulation in humans but isn't apparent in mice until gastrulation has started (Pereira et al. 2011).

Earlier differences in cell specification have been revealed by molecular characterization of mouse and human ES cells and then confirmed *in vivo* from single cell sequencing efforts in mouse (Boroviak et al. 2015; Guo et al. 2010; Ohnishi et al. 2014) and human cells (Stirparo et al. 2018; Yan et al. 2013; Petropoulos et al. 2016; Blakeley et al. 2015). These experiments revealed differences in cell identity in the early embryo. They also pointed to a delay in separating some cell lineages relative to the differentiation of other lineages. These and other differences in early development underscore the power of comparative analysis of human and model organism development in order to craft specific hypotheses that can be addressed in particular model organisms (Rossant and Tam 2017; Shahbazi and Zernicka-Goetz 2018).

Figure 2. Mammalian embryos have different shapes and contact between embryonic and extraembryonic tissues prior to gastrulation.

(A) At the blastocyst stage, the 3 to 4.5 days post fertilization (dpf) mouse embryo and **(B)** 5-7 dpf human embryo are similar, with a surrounding trophoblast (yellow) and forming epiblast (blue) and hypoblast (red). **(C)** As development proceeds just prior to gastrulation, the mouse embryo elongates as the extraembryonic ectoderm (light red) differentiates from the epiblast. The cup-shaped mouse embryo has a small contact area between the embryo proper (blue) and the adjacent extraembryonic ectoderm (pink and grey). **(D)** The human embryo has a disc morphology with extensive contact between the edge of the embryo (blue) and the extraembryonic amniotic tissue (pink). **(E)** Junction of extraembryonic and embryonic cells in mouse (between the dotted lines in C). **(F)** Junction of extraembryonic and embryonic cells in human (between the dotted lines in D). Comparison of E and F shows the larger contact area between these tissues in human.



Another model used in reproductive and developmental biology is the rabbit. Since ovulation is triggered by mating, this allows fertilization to be easily timed. Although rabbits take up much more space than mice compared to the number of embryos produced, rabbits are similarly adaptable to the laboratory environment and have simple care requirements. Moreover, they have disc-shaped embryos at the time of gastrulation, so they resemble human embryos much more closely than mouse embryos. However, rabbit embryos are also very large—about 12 times the diameter of a mouse or human blastocyst. Like many mammals, the rabbit epiblast is contiguous with the trophoblast, so it is not covered by multiple cell layers like mouse and human embryos (Sheng 2015). The rabbit's accessible large and flat blastoderm has allowed for detailed anatomical descriptions during early development, including the first descriptions of what would become known as "Henson's node," a canonical embryonic organizer with homologous (similar) structures in all vertebrates (Viebahn 2001). As with mouse embryos, the size difference when compared to human embryos creates a different embryo geometry that may require striking differences in the mechanisms that pattern the embryo.

Nonhuman primate embryos are the closest to human embryos in their shape, size, and genetics. Several nonhuman primates have been used as proxies for human development. Most work has been done with rhesus macaque embryos, including the isolation of ESCs, the creation of genetically manipulated nonhuman primates, and the analysis of early events in embryo development (Byrne et al. 2007; Chan et al. 2001; Sparman et al. 2009). Most of this focus on rhesus macaques is due to their close relationship with humans coupled with their small size and adaptability to captivity. In spite of these advantages, nonhuman primates like macaques lack many of the features of the other, more robust laboratory animal models. Nonhuman primate embryos are rare, with only one or two generated from a typical mating. Furthermore, there are many ethical issues surrounding the use of nonhuman primates (largely due to the fact they engage and socialize similarly to humans). As a result, research with chimpanzees is no longer supported by the National Institutes of Health (NIH 2015).

Given the changing status of using nonhuman primates in biomedical research, it might become easier to obtain surplus human embryos from IVF treatments than to obtain nonhuman primate embryos. The advent of new technologies, such as single cell RNA sequencing, has allowed for deep data collection from limited numbers of embryos (Nakamura et al. 2016, 2017). Experiments in primate and human embryos (Stirparo et al. 2018; Yan et al. 2013; Petropoulos et al. 2016; Blakeley et al. 2015) may allow direct comparison of gene expression in early stages of development. If the gene expression patterns are similar, limited numbers of nonhuman primate embryos may be used to examine gene expression in later stages of embryogenesis with confidence that they will be close to what occurs in human embryo development.

Nonhuman primate models more distantly related than chimpanzees and macaques may also provide a solution. Recent efforts to develop the common marmoset into a laboratory model have been promising. The common marmoset is small, reaches sexual maturity in about one year, and females typically give birth to twins twice a year. Germline

transmission of an engineered transgene has been achieved in the common marmoset (E. Sasaki et al. 2009) before it was achieved in macaques. While these results are promising, raising these highly social animals in a laboratory is still a work in progress and requires specialized expertise (Orsi et al. 2011).

Despite their many advantages, model organisms do not adequately help scientists understand early human development. Even the use of human cell culture models (discussed in more detail in a later paper) is limited because we can only validate them as far as our current understanding of human development. Only through direct observation and experimentation with human embryos themselves can we confirm the hypotheses built from experiments using animal models.

Technologies for Understanding Human Embryogenesis

Most modern developmental research focuses on understanding how genes control embryo growth and differentiation. Not all genes are expressed in all cells. The term "gene expression" refers to a specific region of DNA that is turned into mRNA, which is then turned into protein. Some of the methods scientists use to detect gene expression in the developing embryo are "*in situ* hybridization," which allows for the detection of expressed mRNA in the developing embryo, and "antibody staining," which allows for the detection of proteins. Examining which genes are expressed where allows scientists to infer their functions or use them as markers to identify particular cell types among similarly shaped cells. With these markers, we can see changes in gene expression before we can see differences in the shapes of cells or tissues, thereby giving us tools to look at embryos with great detail.

Only through direct observation and experimentation with human embryos can we confirm the hypotheses built from experiments using animal models.

Most cells follow a path of specification, commitment, and differentiation. A cell is initially "specified" when it receives instructions to become different from other cells of the embryo. We can detect this difference using molecular markers that measure gene expression. Specified cells can still move into a different path as the instructions change. For example, if a group of cells moves to a new location, their ultimate fate will match their new environmental cues, even if they had begun to change their gene expression to match their original location.

However, as development proceeds, cells lose this developmental plasticity and become "committed." When a cell is committed, it is locked into a set of fates and cannot be changed by altering the environment. These cells then "differentiate," or take on the features and gene expression patterns of that cell's ultimate fate. Understanding this progression from an undifferentiated cell to a differentiated cell requires many different methods to measure and manipulate the system.

Box 2. Scientific Means Used to Visualize and Understand Embryogenesis

Drug treatments – Drugs that affect the functions of specific proteins or metabolic pathways can be used to probe aspects of development. Many specific drugs have been identified that affect specific signaling pathways.

Embryo manipulation/surgeries – The rearranging or removing of embryo parts can help to identify the developmental potential of different parts of the embryo or identify important signaling centers that organize the embryo.

Gene knock-down – Various methods that decrease the expression or function of a gene product and reveal the function of a gene. These methods are distinct from gene mutation.

Gene mutation – The activation or inactivation of specific genes that may result in developmental changes, revealing the function of that gene. Methods used include homologous recombination in mouse ESCs and the use of targeted genome editing tools.

***In situ* Immunohistochemistry –** Reveals the expression of specific proteins with cellular or even subcellular resolution in intact, fixed embryos. By examining which cells express specific proteins or where the proteins are localized within specific cells, we can predict gene function or examine the changes that result from different experimental manipulations.

RNA *in situ* hybridization – Reveals the expression of specific RNAs with cellular resolution in intact, fixed embryos. By examining which cells express specific mRNAs, we can predict gene function or examine the changes that result from different experimental manipulations.

Transgenic expression – Transgenes are DNA from another source inserted into the genome. They require promoter DNA sequences to control where and how much the transgene is expressed. This promoter sequence can then direct expression of different mRNAs depending on the application. Reporter sequences such as fluorescent proteins can be used to tag genes and reveal their expression patterns in living embryos. Wild type genes can be overexpressed or expressed in different cells to determine what effect they have on development. Fluorescently tagged versions of wild type genes can be used to reveal the location of a protein within a cell.

Video microscopy – Direct observation of embryos over time that allows us to record how cells move, divide, and change shapes during embryo development. Coupled with transgenes that report on specific aspects of the state of cells, this is a powerful method for determining how embryos develop.

One primary way that scientists examine embryo development is to watch them grow. New microscope technology and computer resources have transformed how we can watch what happens in developing embryos. One of the features that made the worm, *C. elegans,* a powerful developmental model organism is the simple organization and an "invariant lineage," or a largely predictable series of cell divisions and differentiation steps that gave rise to each cell of the adult. This "lineage map" was made possible by investigators who watched embryos develop repeatedly, tracking the fate of each cell over time (Sulston et al. 1983; Deppe et al. 1978).

Vertebrate embryos do not have the invariant lineage of *C. elegans,* but recording what happens during development is still a powerful way to understand what is happening in the developing embryo. Using fluorescent dyes or introducing transgenes, scientists label cells and take pictures of embryos over regular time intervals. These pictures can then be assembled into time-lapse movies of development and analyzed repeatedly to describe the movements and

shape changes of tissues. Even individual cells can be visualized, depending on how the specimen is labeled. This technique requires multiple embryos to be imaged so that the differences and common elements can be identified, making the data generalizable to all embryos of that organism.

Embryos are extremely dynamic. Having a time-lapse record is comparable to having the same number of fixed embryos as you have frames of your time lapse. In fact, a time-lapse is even better because scientists can track the behaviors of single cells over time. This dynamic aspect of development is already being used to improve the IVF transfer rate in humans.

Researchers also try to turn genes on and off by isolating or creating mutations that alter their functions. Scientists then examine the consequences of changing gene activity to address questions such as: Is development disrupted, and if so what happens in the embryo? Are there changes in the expression of other genes that lead some cells to have different cell fates than what they originally had? Are there differences in the way cells move in the embryo? The results are often surprising. Many genes that are hypothesized to have important functions in particular processes have turned out to be required for something else entirely. In other cases, losing a gene has no obvious effect on the organism's development at all. Many inbred lines of mice have been developed in which all members of the strain have identical DNA. The phenotypes observed for gene mutations within a particular mouse strain are usually consistent, but they are often different when bred into a different genetic background (Doetschman 2009), requiring detailed analysis in multiple backgrounds to really understand the requirement for a particular gene. We lack inbred strains for humans, but the more scientists compare the genetics of humans with model organisms, the more they find that there are humans who should have debilitating diseases but appear to be perfectly normal and relatively healthy (R. Chen et al. 2016; Sulem et al. 2015). This highlights the genetic variability of humans and means that it will be difficult to draw conclusions about all humans from the few embryos that exist in collections.

The Developing Human Embryo

Early Embryo Development: Fertilization through Gastrulation

Like other mammals, human embryos begin development inside of their mothers. Following the fusion of the egg and sperm, the resulting one-cell embryo, or zygote, undergoes a series of cell divisions that result in a ball of cells called a morula (approximately 3 dpf). Up to this point, all of the cells hold the same potential (equipotential).

As the number of cells increases, eventually some cells begin to specialize, resulting in the formation of the blastula (5 dpf). This is the first of many steps that will result in different types of cells. Cells inside the blastula will form the embryo and some extraembryonic tissues. Cells on the outside will become extraembryonic tissues. Fluid is secreted into the center of the embryo to form a hollow space called the blastocoel. This blastula stage embryo is usually the point by which embryos are transplanted following IVF. After this

stage, we know much less about how human embryos develop, and we rely more on comparisons with model organisms.

During implantation, the blastula stage embryo embeds into the wall of the uterus. This process requires interaction between the embryo and the uterine lining, resulting in the proliferation of the uterine cells that surround the developing embryo with maternal tissue. This maternal-embryo interaction will become more and more elaborate over time. Reproducing the maternal component of this interaction is currently one of the greatest challenges of *in vitro* embryo culture.

Around 7 dpf, the internal cells of the embryo are still dividing to form the extraembryonic ectoderm and hypoblast as well as the embryonic blastodisc or epiblast that will form the embryo proper. At this stage, the epiblast is still "undifferentiated," and its cells therefore still have the potential to make any type of cell in the embryo. Up until gastrulation, which begins at 15 dpf, the epiblast has the capacity to split and form two complete embryos inside the same set of extraembryonic tissues, thereby forming identical twins. When gastrulation begins, the embryo undergoes a dramatic reorganization to transform the flat disc of cells into a multilayered structure. This reorganization continues as the cells begin forming a tube with three layers—the endoderm on the inside, the ectoderm on the outside, and the mesoderm in the middle.

Each of the three layers becomes subdivided, responding to cues from their neighboring cells. These subdivisions undergo their own morphogenetic movements to begin forming the rudiments of different tissues and organs. The tissue and organ rudiments continue to undergo morphogenesis to take on their appropriate shapes. Local cues direct cells to change their shapes and move relative to each other in order to shape each tissue. Some of these cues come from within the tissue or organ itself, and some of them come from neighboring tissues, which underscores the large degree of coordination of these developmental processes. What is particularly important at this point is the coordination in space and time of cell and tissue shape in the developing embryo. If the right cells are not in place to produce a signal and other cells are not in place to receive it, things go wrong and embryonic defects occur.

The Developing Central Nervous System: An Example of Tissue Formation and Differentiation

To demonstrate the roles of these different processes that pattern and shape a tissue, we will focus on the developing central nervous system (CNS). This information comes from experiments performed in model organisms to understand the roles of specific signaling molecules and the cellular rearrangements that shape the CNS in different organisms. Scientists believe that the same events most likely occur in human embryo development, although with some variation. But scientists are unlikely to know exactly how things happen in the human embryo until embryos can be directly observed. However, the data obtained from experiments in model organisms do allow scientists to focus their

examination of human embryos and limit the number of embryos required to answer specific questions.

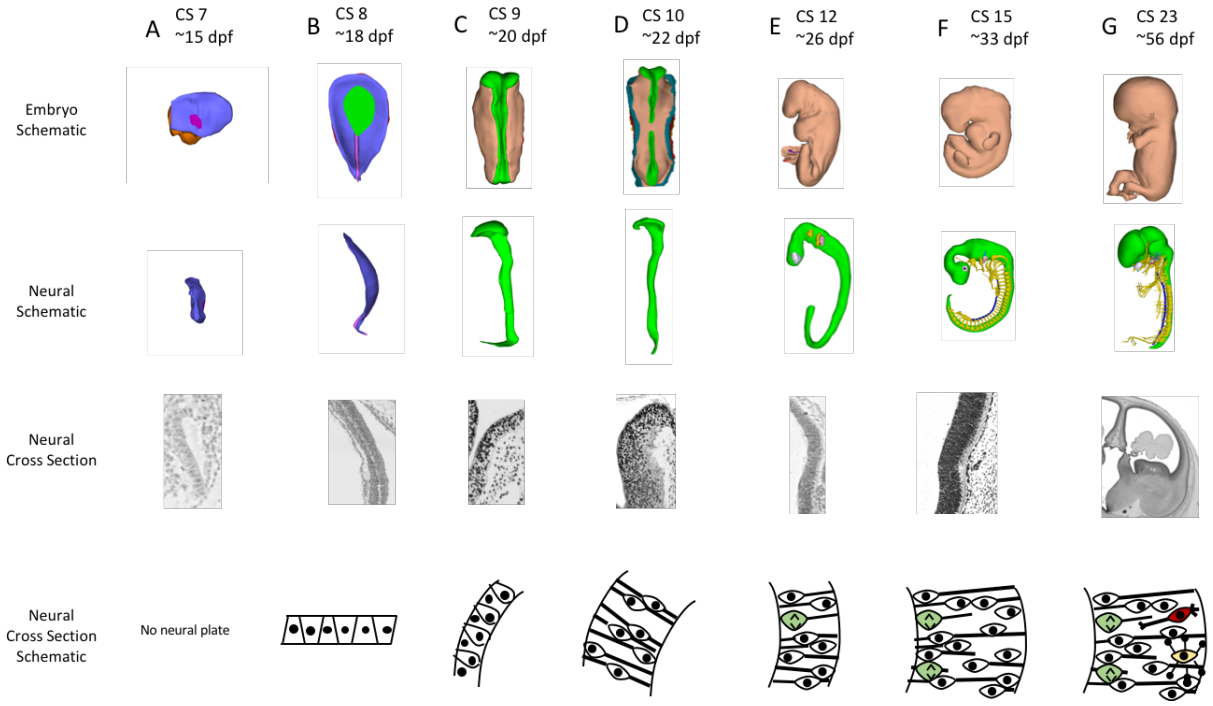
The CNS arises from the ectoderm, the cells that will be on the "outside" of the embryo. During gastrulation, the cells of the ectoderm begin to differentiate, following multiple signals that direct cells to do so. These signals can operate at short or long range in the embryo. Most signal pathways are subject to regulation by other factors that may limit or potentiate their effects depending on the cellular context. For example, in all vertebrate embryos studied, signaling molecules called bone morphogenetic proteins (BMPs) instruct cells of the ectoderm to adopt different cell fates (De Robertis and Kuroda 2004; Sasai and De Robertis 1997). The amount of BMPs or the time of exposure to BMPs instructs the cells to take on specific fates in conjunction with other signaling pathways that reinforce and sharpen boundaries between different domains. Cells that see high BMPs will become the epidermis of the skin. Cells that see low BMPs will become neurons in the brain and spinal cord. Cells in between form specialized cells called the neural crest and placodes, which make many different parts of the embryo, such as parts of the eyes, ears, and pigment cells in the skin. The gradient of BMP activity is established in part by localizing production of BMPs to some cells and producing BMP antagonists to counter the action of these BMPs (Holley and Ferguson 1997; Little and Mullins 2006). The complex interplay of the sources and sinks of BMP activity means that the details of geometry are important.

While scientists have learned a great deal about the role of BMPs and their antagonists in this patterning event in many different organisms, the source of the BMP signal and the precise shape of the BMP gradient likely depend on the shape and size of the embryo (Figure 3). Since no animal models have the same shape and size as human embryos, our understanding of BMP signaling and its impact on cell fates in human embryos is limited.

After the neural plate develops at 15 dpf (Figure 3A), its shape begins to change to form a tube that will become the brain and spinal cord. This process requires a complex set of cell shape changes that extend the neural plate along the rostral-caudal (nose-to-tail) axis and then raise the edges of the neural plate and curl them toward the middle of the embryo to meet in the middle. To execute this movement correctly, cells need to have appropriate positional identity, which means they need to know where they are along the proximal-distal (middle-to-side) and rostral-caudal axes. These cell movements are directed in part by the planar cell polarity (PCP) pathway. This pathway manages the orientation of cells to ensure that they can coordinate their behaviors and reshape the tissue. Originally identified in the fruit fly, *D. melanogaster*, homologous PCP pathway genes have been shown to function in rearranging cells in the neural plate as well as shaping other tissues such as kidney tubules (Copp, Greene, and Murdoch 2003a, 2003b; Carroll and Das 2011).

Figure 3. The dynamic changes in the formation of primitive neural tissue during embryogenesis.

(A) The ectoderm (blue at 15 dpf schematic) separates from the other germ layers. **(B)** The neural plate is induced from the ectoderm around 18 dpf and consists of a flat single layer of cells (cross section schematic). **(C)** Following induction of the neural plate (green) curls up to form a neural tube over the course of several days. **(D)** As closure initiates in the middle of the neural tube at 22 dpf, the neural plate at the anterior thickens, with some cells piling up on the others as they rearrange to close the anterior neural tube (cross section). **(E)** At 26 dpf, the neural tube is closed, and the cells form a pseudo-stratified epithelium (cross section). Dividing cells are limited to the inner ventricular surface (green). They are mostly a single layer, and the nuclei have to be in different depths to pack tightly in the neural tube. **(F)** By 33 dpf, cells are leaving the inner layer of the neural tube. Regional subdivisions for the forebrain, midbrain, and hindbrain become obvious. **(G)** By the end of embryogenesis, the brain has undergone dramatic changes in morphology and organization. The embryo schematic, neural schematic, and cross sections are derived from de Bakker et al. (2016).



Note: CS – Carnegie Stage

The neural plate at this stage, approximately 18 dpf (Figure 3B), is one cell thick with fairly tall and thin cells. These cells change their shapes so the top side of each becomes smaller and the bottom side facing the inside of the embryo becomes thicker. This small change of each cell drives the shape change of the tissue as a whole and causes the sheet to roll up and form a tube (Figure 3C-D). There are more specific changes that occur at different points, and specific aspects of the tube diameter and shape depend on the position along the rostral-caudal axis.

Beginning during neurulation and continuing after neural tube closure at 26 dpf (Figure 3E), the cells of the neural tube proliferate. Cell division occurs initially in the plane of the tube to generate more neural stem cells. As they continue to divide, some division will occur in an inside-out direction, with one cell on the inside making another stem cell and the other cell leaving the inner layer to make other more specialized cells in the brain.

The decision to remain as a stem cell or to divide and begin populating the brain is directed by the Notch/Delta signaling pathway (Zhang, Engler, and Taylor 2018; Pinto-Teixeira and Desplan 2014). Originally identified in the fruit fly, *Drosophila melanogaster*, this pathway is used repeatedly during development to establish boundaries between domains within or between tissues, or to establish unique cell identities from within a uniform field (Lewis 1998; Sjoqvist and Andersson 2017). As more and more cells leave the inner layer of the neural tube, they begin to organize into the layers of the forming brain and spinal cord. They migrate to specific positions, initially forming radial glia and other supporting cells, and then begin to differentiate, with the first neurons being produced around 45 dpf (Bystron, Blakemore, and Rakic 2008; Stiles and Jernigan 2010). Patterning within the CNS along the dorsal-ventral axis requires coordination of Sonic Hedgehog signals from the ventral side of the neural tube and Wingless and BMP signals from the dorsal side. These pathways are used to pattern other tissues as well, but the specific cell types that arise in the spinal cord depend on the amount of each signal they receive (Le Dreau and Marti 2012; Ulloa and Marti 2010).

From the simple organization of the neural tube at 33 dpf to the end of the embryonic period at 8 weeks, we get an incredible elaboration and separation of the neural tube into distinct zones (Figure 3F-G). The cells of the cerebral cortex, the elaborate structure that differentiates our brains from most other mammals, are still very simple. Following the embryonic period, cells of the brain will continue to proliferate and differentiate into the diverse cells that make up the postnatal brain. How these cells become the diverse cells of the developing brain is the subject of a great deal of research, and it is precisely this step that makes humans different from other animals.

Gaps in Human Development Knowledge and Where to Find Answers

Given what we know from animal models, scientists are now trying to determine how human embryo development differs from other organisms. However, there is a considerable gap in our knowledge between implantation and 8 weeks. By allowing access to extrauterine embryos, IVF has permitted a detailed examination of human embryos and

a clearer understanding of the morphology of normal and abnormal human development from fertilization to 5 dpf (DeCherney and Barnett 2016; A. A. Chen et al. 2013; Fechner and McGovern 2011). Unfortunately, due to the limited number of human embryos available for research and the small number of laboratories willing to undertake this work, our knowledge of human embryo development is lacking.

Recent work on human development has focused on the early steps, with an interest in nuclear reprogramming, which is the ability to return a differentiated cell to an undifferentiated state similar to a cell in the early embryo. This was the method used to clone Dolly the sheep and other animals (Tachibana et al. 2013; Wilmut et al. 1997). It was also used to produce induced pluripotent stem cell (iPS), which are differentiated cells (such as skin or blood cells) reprogrammed to function similar to ESCs. (Takahashi et al. 2007; Takahashi and Yamanaka 2006; Yu et al. 2007). Research using these methods has focused on the early steps of cell differentiation and seeks to answer the question, "how do cells achieve and maintain a pluripotent state in culture?" (Takashima et al. 2014; Smith 2017).

Using state-of-the-art single cell RNA sequencing technologies, scientists have revealed differences between mouse and human embryo early cell fate decisions as early as the beginning of the blastula stage (Blakeley et al. 2015; Stirparo et al. 2018; Guo et al. 2010; Boroviak et al. 2015). These investigations are well within the 14-day limit, but differences between cell fate decisions and pluripotency regulation in humans and mice remain unknown. It is likely that as we look closer at human embryo development, more differences will emerge. With carefully chosen questions and robust preliminary data from animal models, scientists are now considering the technical possibility of extending the period of human embryo culture.

Determining and Defining Stages of Human Embryo Development

To determine an appropriate stage to stop human embryo culture, scientists need to consider the ethical limits as well as the presence of robust features in the embryo that will allow for accurate determination of the embryo's developmental stage. When deciding on a stage to stop, there must be an unequivocal ability to identify morphological features that are clear hallmarks of a specific stage. The Carnegie staging series describes morphological features of developing human embryos and is used to compare embryos of different vertebrate animals. These descriptions rely on features that can be observed in the developing embryo without dissection or using molecular detection strategies, and they serve as an excellent guide for what we would want to consider for evaluating cultured human embryos.

Several challenges exist in determining the age of the embryo based on timing alone. First, the precise timing of fertilization is usually unknown unless the embryos are derived from IVF. While Carnegie stage samples have temporal ages assigned to them, embryonic ages are approximate for most

It is likely that as we look closer at human embryo development, more differences will emerge.

specimens that represent stages after 14 dpf. Second, variation in the rate of embryo development also creates discrepancies in the chronological age and developmental age (O'Rahilly and Müller 2000 2010). Third, the features we would use for stage determination must be robust and detectable without disrupting embryo development. Unfortunately, our ability to visualize the embryo will depend on the details of its culture requirements. For visual inspection of the embryo, extraembryonic tissues may obscure the developing embryo. In such a case, we may need to rely on features such as the presence of circulation in the extraembryonic tissues beginning with the beating heart tube around 21 dpf. If culture conditions do not require substantial extraembryonic tissue growth, this could allow for detection of other clear measures of embryo development.

In spite to these difficulties, several factors could be helpful in determining relevant developmental stages. One of them is the appearance of major structural features during the first four weeks. For example, somites, or paired blocks of mesoderm that form on either side of the developing spinal cord, appear at 20 dpf. The anterior somites form first, with additional pairs being added regularly as development proceeds. In model organisms, this regular addition is predictable and used as a marker of embryo age in zebrafish, for example (Schroter et al. 2008).

The shape of the embryo could be another feature used to measure developmental stages. The limbs form initially as buds that protrude from the surface of the side of the embryo. The appearance of the forelimb buds at 26 dpf or the hindlimb buds at 28 dpf provide an obvious silhouette, even if visibility of the embryo is limited by the culture conditions. While these features are robust indicators for morphological hallmarks, some features should be used with caution, such as neural tube closure at 22 dpf, since many of the questions we may have regarding human embryo development are likely to affect neural tube morphogenesis, such as the genetic and environmental causes of spina bifida. If morphological hallmarks are used to set later limits of development, it would be prudent to identify multiple distinct features that are unlikely to influence each other to ensure that the hallmark is not confounded by any experimental manipulation. In addition, sentience or the ability to feel pain are major ethical concerns surrounding the extension of human embryo culture. Whatever measures are established, it would be prudent to have a clear understanding of the correlated neural development. Further, there should be knowledge of the outward measures of developmental age and other aspects of embryo development under the optimized culture conditions.

What Can We Learn from Culturing Embryos at Later Stages?

What does *in vitro* culture of human embryos promise? Many causes of human birth defects and implantation difficulties likely arise during the first 4 weeks of development. During this time, the basic plan of the embryo is established, the rudiments of many organs develop, and a complicated interaction between the embryo and the uterus forms the placenta to support embryo growth.

In vitro culture allows for the observation and testing of embryo function within these stages. Culture up to 14 dpf encompasses early cleavage and formation of the blastula up to

gastrulation. Defects in this stage are likely to produce inviable embryos and lead to pregnancy failure. This is important knowledge to acquire, as many pregnancies end in spontaneous abortion. The embryo begins to interact with the uterus at about 6 dpf, so examining the interaction between the embryo and uterine tissue proxies in culture may also reveal some of the causes of implantation failure.

To culture embryos beyond this stage would require investigators to mimic these interactions *in vitro*. Figuring out how to do this would reveal the nature of these interactions and the potential sources of infertility and spontaneous abortion that arise around implantation. The most obvious developmental defect that arises around implantation and later is the formation of conjoined twins. Extending *in vitro* culture past 14 dpf to 21 dpf opens the door to investigating defects in neurulation, such as spina bifida (Copp, Greene, and Murdoch 2003b). Likewise, the heart is beginning to loop and contract, and embryonic defects in heart development will first manifest at this time. Defects in heart formation are some of the most common human birth defects with a rate of just under 1% of live births (Reller et al. 2008). If the heart tube is not patterned correctly, later folding and fusing to form the four chambered heart will also be impacted (Gittenberger-de Groot et al. 2014).

Extending in vitro culture past 14 dpf to 21 dpf opens the door to investigating defects in neurulation, such as spina bifida.

Extending *in vitro* culture to 28 dpf would allow for the observation of heart looping and the beginning of heart septation into the right and left sides. Twenty-eight dpf would also allow for the observation and testing of the formation and patterning mechanisms of the face, limb buds, and many other organs whose rudimentary structure is formed at this time. In addition, the neural crest migrates out from the dorsal neural tube during this period. These cells migrate to many different parts of the body and form or contribute to different structures in the developing embryo. Examining neural crest migration will help understand the origin of diseases such as cleft lip and palate, hereditary deafness, and Hirschsprung's disease (a congenital defect related to intestinal development), among many others (Trainor 2016; Noisa and Raivio 2014). With each time extension of embryo culture, more organs and the developmental processes that regulate their formation can be investigated to understand the genetic and environmental causes of human birth defects.

We cannot understand human development without examining human embryos. By building robust preliminary data from model organisms, such as nonhuman primate modeling, scientists can focus on the questions that are most important to answer when using human embryos for research. Furthermore, this would limit the number of human embryos used in research. Choosing the right technology and techniques to

With each time extension of embryo culture, more organs and the developmental processes that regulate their formation can be investigated.

investigate the developing embryo, such as high-resolution video microscopy and single cell RNA sequencing, will allow for robust data sets that can be examined by multiple investigators. This infrastructure coupled with vigorous oversight and clear limits on the time of embryo culture would allow us to understand this human embryo development.

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