HOW TO CONDUCT ETHICAL RESEARCH ON SHEEFS: BIOLOGICAL BACKGROUND, THE CLASSIFICATION, AND RECOMMENDATIONS FOR GUIDELINE DEVELOPMENT ON THESE NEW SYNTHETIC EMBRYOS

BY

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LIST OF ABBREVIATIONS

BMP4: Bone Morphogenic Protein 4

CNS: Central Nervous System

EMROs: Embryo Research Oversight

ESCROs: Embryonic Stem Cell Research Organizations

ESCs: Embryonic Stem Cells

hESCs: Human Embryonic Stem Cells

HFEA: Human Fertlisation and Embryology Act

hPSCs: Human Pluripotent Stem Cells

IBC: Institutional Biosafety Committee

iPSCs: Induced Pluripotent Stem Cells

ISSCR: International Society for Stem Cell Research

IUCAC: Institutional Animal Care and Use Committee

IVF: In Vitro Fertilization

NAS: National Academy of Sciences

NASEM: National Academy of Sciences, National Academy of Engineering, and Institute of Medicine

NGO: Non-governmental organization

PS: Primitive Streak

PSCs: Pluripotent Stem Cells

SCNT: Somatic Cell Nuclear Transfer

SCRO: Stem Cell Research Oversight

SHEEFs: Synthetic Human Entities with Embryo-like Features

ABSTRACT

New forms of Human Cell Culture Models are able to recapitulate human embryogenesis for up to 14 days of culturing in laboratory conditions. And with this advent, there is a potential in these developmental technologies to provide new types of systems for drug testing, stem cell technologies, and regenerative medicine, to name a few applications. Since these synthetic embryos (also known as, synthetic human entities with embryo-like features or SHEEFs) are able to present features of a human embryo around the same time, should they be considered human embryos or be subject to embryo regulations? SHEEFs, when created in laboratory by abiding by guidelines formed by international committees like the ISSCR (International Society for Stem Cell Research), or by following the recommendations by the National Academy of Sciences, National Academy of Engineering, Institute of Medicine (NASEM) on human embryonic stem cells, have the potential to be effectively used as a suitable replacement for animal models, or embryo-destructive research. But, at the same time, using SHEEFs unethically, to implant them in animals, or create chimeras in laboratory conditions in combination with animal extra-embryonic tissues, pose an ethical impasse. Development of proper guidelines on the creation and use of these human embryonic cell culture models is necessary to offer research scientists a clear set of rules and to remove confusion regarding research on them. This thesis is an attempt to categorize SHEEFs, discuss their developmental potential, and by envisioning some future applications, devise a set of recommendations that will help form the regulations.

INTRODUCTION

To tackle developmental diseases and infertility, and learn more about early human development, scientists study embryos. New human cell-culture models that mimic human embryogenesis in vitro are being studied and developed. One such example is a group of organized human embryonic stem cells (hESCs) that were able to mimic canonical embryogenesis till the 15th day (Warmflash et al. 2016). These self-organizing hESCs present as a potential alternative to embryo-destructive research and to using animals for research. Currently, there is uncertainty regarding how these entities should be understood, how they relate to human embryos, and the ethical guidelines that should inform research on them. There is a need for deliberation to determine how research on these entities should proceed.

The 14-day rule that limits research on embryos up until the 14th day after fertilization originated from two major sources: 1979's report of the Ethics Advisory Board to the department of Health, Education, and Welfare in the U.S. and the Warnock Committee of Inquiry into Human Fertilisation and Embryology in the U.K. (Ethics Advisory Board 1979; Warnock 1988) It is a widely accepted rule that is applied to intact human embryos and that says any research on them, no matter what the purpose (assisted reproduction or scientific advancement), should be terminated after 14 days or the appearance of a primitive streak (whichever occurs first), and the embryos used must be destroyed thereafter. This diplomatic compromise, in different versions, has since been adopted in

many countries: in some, as part of laws or regulations and in some, as an ethical guideline (as in the United States). This compromise arose, in part, to settle the dispute among scholars and scientists holding competing views about the moral status of an embryo at various stages of its development (Devolder and Devolder 2010; Maienshein 2005; Mieth 2000).

It was not possible to maintain human embryos past 14 days post fertilization until the last two years. Human embryos are destroyed after 14 days of development in cell-culture conditions or after the appearance of a primitive streak, which indicates the incidence of gastrulation in them, to avoid violating the 14-day rule.

"Embryo research" is a broad term that includes a variety of research done on embryos in various settings such as in biomedical companies, academic institutions, and fertility clinics. Some research on embryos is conducted pre-implantation[1] (Hill, 2019), after creating them in lab via in vitro fertilization (IVF), after which the embryos are transferred into a womb. In this thesis, embryo research solely refers to ex utero research[2] done on embryos in laboratory settings that are not intended to be implanted into a uterus. This kind of research also involves creating embryos in the laboratory by techniques such

[1] Embryos are typically implanted on day 8 after in vitro fertilization
[2] Or, "embryo destructive research" as used by Tollefsen in his paper (Tollefsen 2001).

as somatic cell nuclear transfer (SCNT)[3,4], in-lab gamete fertilization[5], or using embryos that were donated[6] for research (embryos that are left over from IVF procedures).

In addition to research on human embryos, a variety of entities have been created using ESCs that mimic various aspects of human embryo development. Some of the types of these entities are synthetic embryos created in a laboratory by growing human pluripotent stem cells (hPSCs) in a dish. hPSCs are those cells that can develop into different cell lineages in the human body such as neurons, cardiac cells, epithelial (skin) cells etc. SHEEFs are made out of these cells and, hence, are not totipotent. Human embryonic stem cells (hESCs) are cells that are extracted from human embryos and are totipotent which means that in addition to possessing the capacity to develop into any cell lineage they also have the ability to further divide and differentiate to form an embryo and a placenta (Science Reference Services, LOC 2018). However, after a few cell divisions hESCs lose their totipotency. Synthetic embryos created in the laboratory are usually comprised of ESCs. These are called gastruloids or embryoids. Synthetic embryos are known as gastruloids because of their capacity to reach gastrulation stage in canonical[7] embryogenesis and are made up of hESCs.

[3] That was used to clone Dolly sheep over two decades ago.
[4] Therapeutic cloning, used for transplants, is of two types: SCNT and Pluripotent stem cell induction or Induced pluripotent stem cells. Therapeutic cloning is an actively researched area, but hasn't been used in medical practice, as of April 2017 (NIH, 2017)(Vogel 2014).
[5] In lab gamete fertilization is in its nascent stages. Female gametes or eggs (or ova) can be created in a lab and be fertilized with stored sperm – this is not a process that is routinely used to create embryos for research. The most common way to procure embryos for research is by using the ones that are donated for research after IVF procedures. This is also called "therapeutic cloning". SCNT is a type of therapeutic cloning, the other type being induced Pluripotent Stem Cells (iPSCs).
[6] With informed consent from the donors.
[7] "Canonical", for the purposes of this thesis, is used as a synonym for "typical". "Canonical embryogenesis", thereby, being synonymous with "typical process of an embryo's growth".

There are other types of entities created in vitro, known as organoids, that do not possess totipotency; they are tissues made up of cells belonging to one type of cell lineage, e.g., brain[8]. Brain organoids are used to study incidence of neurological diseases like schizophrenia and Alzheimer's disease, whereas gastruloids are used to study the stages of embryonic development in vitro (e.g. formation of a blastocyst, splitting into three layers). Studies on gastruloids also focus on finding ways to grow human embryos in vitro by experimenting with different extra-embryonic markers (markers that are received from the mother via placenta). One of the notable markers used in such studies is BMP4 which contains the gene Brachyury (T/Bra) (Gastruloid 2019) that marks the primitive streak and the site of gastrulation. Such studies administer extra embryonic markers to the gastruloids in varying dosages and gradations, and this technique is called "micropatterning" (Susanne C. van den Brink et al. 2014).

Till now gastruloids have been grown in vitro till 14 days and most recently a group of scientists (Warmflash et al. 2016) were able to grow these till 14 days and observe gastrulation, which is the division of cells in the blastocyst into three distinct layers – endoderm, mesoderm, and ectoderm. They were also able to observe the primitive streak on the 14th day. In a different lab, the same was accomplished using mouse pluripotent stem cells (Susanne C. van den Brink et al. 2014). In some other labs, hESCs were used but micropatterning[9] wasn't integrated into their culturing (Deglincerti et al., 2016;

[8] Known as "brain" or "neural organoids". "Neural organoids, also known as cerebral organoids, are hPSC-derived three-dimensional in vitro culture systems that recapitulate the developmental processes and organization of the developing human brain." (Stem Cell Technologies)
[9] Micropatterning (which can also be known as "spatial confinement") will be explained in the next section in more detail.

Itskovitz-Eldor et al., 2000; Shahbazi et al.[10]); these were also successfully grown till 14 days, but gastrulation was not observed. After 14 days, gastruloids in Warmflash and colleagues' experiments. (henceforth, the gastruloids such as those grown in Warmflash and colleagues' lab will be referred to as SHEEFs – Synthetic Human Entities With Embryo Like Features) were destroyed to abide by the 14-day rule as the embryonic stem cells (ESCs) that were used to culture the gastruloids were of human origin and were able to mimic a human embryo at that stage. Although these entities were not a product of fertilization of gametes but were derived from human embryonic stem cells and eventually developed to appear like 14 day old embryos, it is uncertain as to how they must be dealt with; and this ambiguity in handling them was noted by the scientists.

These entities are not human embryos and thus are not necessarily subject to the same research guidelines that apply to human embryos. In the immediately following section, differences between embryos and SHEEFs will be further discussed. While SHEEFs are not an urgent regulatory concern given their inadequate replication of a human embryo, there is a need for lucid guidelines to make research on them more seamless. In this thesis, I propose some guidelines for their regulation.

In this thesis, I propose that SHEEFs [i.e., synthetic embryonic entities formed in-vitro out of human pluripotent stem cells (hPSCs[11])] should be left out of the 14-day rule and

[10] "In vitro attached human embryos" were used in the Deglincerti et al paper. Shahbazi et al. cultured human embryos donated specifically for their project in vitro[10]. In Itskovitz-Eldor et al. used human pluripotent stem cells.

[11] Human embryonic stem cells are a type of human pluripotent stem cells. hPSCs possess totipotency in the first few cell divisions (discussed later on page 17). But lose totipotency a few cell divisions later after which they are known as hPSCs.

that research on them should be governed by guidelines specific to SHEEFs. I also propose that SHEEFs should not be grown *in vitro* if the purpose of the study is to implant them into animals or humans for gestation, as this can raise some ethical concerns similar to those raised by human cloning and human-animal chimeras. Experiments that aim to create SHEEFs in a way that they possess the form and shape of a human embryo but deviate from canonical development in a critical way, e.g., they lack vital organs or possess multiple copies of one organ, may also raise some ethical questions. Observing the development of SHEEFs in artificial conditions such as cell-culture conditions or artificial wombs[12] would be a very important step forward in developmental biology. But growing SHEEFs to harvest organs, like human-animal chimeras are being developed to be used, is ethically problematic and in order to maintain the trust of the public, such uses of SHEEFs should be carefully evaluated. Guidelines on creation of such canonical variants of SHEEFs and the continuation of growth of SHEEFs *in vitro* should be established after careful deliberation by national and international organizations such as the ISSCR, ethics committees such as Embryo Research Oversights (EMROs), and Embryonic Stem Cell Research Oversights (ESCROs), scientific organizations such as the National Academy of Sciences (NAS), non-governmental organizations (NGOs), and other professional groups. Depending upon the scientific objective of the experiment in which SHEEFs are used, there may be a need for institution-based stem-cell research oversight committees to evaluate whether the study justifies the use and disposition of SHEEFs. As researching on human cell-culture

[12] The term "in vitro" when used to describe the growth environment of SHEEFs post 14 days should be understood as comprising of both cell-culture conditions and artificial wombs insofar as their successful creation is possible. The prospect of growing SHEEFs in artificial wombs will be discussed in subsequent chapters.

models is an international practice, guidelines on SHEEFs should be established globally. Similar to the establishment of the 14-day rule and the recent recommendations on the use of the CRISPR/cas9 gene editing tool, each country should encourage nation-wide deliberations on this topic.

SHEEFS – WHAT IS KNOWN

In this thesis, I explain the current advances in human embryonic stem cell technologies, and I also explain how new human embryonic cell culture models such as SHEEFs differ from human embryos. Some such differences between the two entities (SHEEFs and embryos) will be provided in this preface, so readers can look for them as the thesis progresses. Also, as the research on SHEEFs is considered nascent, some advances in this field of hESC technologies are assumed in this thesis, and the most ambitious of such research studies are mentioned, here, only to serve as concerns which the recommendations can address.

The most notable difference between SHEEFs and embryos, to me, is their inherent potential to become a person. This inherent potential may not seem like a valid scale by which these two entities can be measured, because not much is known about SHEEFs' potential. Nevertheless, I regard embryos as entities that have this said inherent potential, because compared to them, SHEEFs are just aggregates of hESCs. Indeed, SHEEFs are aggregates of hESCs or are human embryonic cell culture models that have an added advantage over the previous hESC culture models, in that they are designed to develop

spatially similar to human embryos through micropatterning. As should be clear from reading the works of Warmflash and his team (Warmflash et al. 2016), SHEEFs are hESC cultures that developed a PS and exhibited the three types of cells (endodermal, mesodermal, and ectodermal), but in no way were referred to as "intact embryos" by the researchers. SHEEFs are better models of human embryos than some previously created models (e.g. the synthetic embryos created by (Shahbazi et al. 2016a)), but they, too, fall short of mimicking human embryos completely. Some reasons for that are mentioned in a later article published by Warmflash et. al.: SHEEFs do not have the orientation concept that human embryos do, that aligns the gastruloid's contents in a certain direction (rostral-caudal), the PS in SHEEFs is formed in a circular fashion whereas in a human embryo, the PS is formed in the caudal midline, and SHEEFs lack many markers that are usually found in human embryos (Sapna Chhabra, Lizhong Liu, Ryan Goh, Aryeh Warmflash 2018). These differences, at the stage of 14 days, may not seem so significant, but in biological terms, what this essentially translates to is that: SHEEFs might not develop into a fetus that has organs arranged in a certain direction, instead, the organs may develop inside a circular entity without any sense of the head to tail orientation. SHEEFs also may never develop these organs if they lack markers or cell progenitors, inhibiting the cells in SHEEFs from differentiating into various cell-lineages. What this should broadly render to the readers is that SHEEFs should not be considered as entities capable of mimicking further (beyond 14 days) stages of human embryogenesis. SHEEFs are hESCs that have been micropatterned in laboratory conditions to recapitulate early stages of human embryogenesis. Beyond this, whether SHEEFs are designed to mimic the developmental stages that occur after 14 days is up to the research scientists

conducting experiments on SHEEFs. In essence, this should be understood as the researchers molding the growth of SHEEFs into entities that look like human embryos. And, it follows that SHEEFs, in and of themselves, do not possess capacities or capabilities to become entities that appear like human embryos. Therefore, SHEEFs do not possess an inherent potential or moral status.

It may also be derived that SHEEFs grown in Warmflash and colleagues' lab may not develop by themselves after 14 days if the researchers do not intervene by geometrically confining their growth, and by adding other extra-embryonic markers crucial to human embryogenesis. Even if the researchers design experiments to allow for SHEEFs to grow like human embryos, beyond 14 days as well, there is no guarantee that SHEEFs may successfully recapitulate human embryos' growth. Similarly, there is also no assurance that SHEEFs may integrate with animal cells if implanted into non-human animals, let alone contribute to the successful growth of human organs inside animals. SHEEFs, therefore, may not contribute more effectively to the field of artificial organs than to the field of studying basic developmental biology.

Warmflash and colleagues used hPSCs to create SHEEFs in which an interplay of different signals (this interplay of signals is discussed more in detail in following sections) is used to harness the self-organizing patterning in the cells. Their future studies might be designed to understand the cell-cell signaling that contributes to the generation of different cell-lineages (paracrine signaling in early development), and other basic developmental biology questions like such. In their article, Aach et. al. describe the

hESCs in Warmflash and colleagues' experiments as a "simple form of SHEEF", and suggest that advanced forms of SHEEFs may be capable of developing features that are be more morally concerning (Aach et al. 2017a, 3). For instance, they theorize that SHEEFs may bypass certain moral checkpoints (such as a PS) to develop features that mimic later stages of human embryogenesis (such as developing a heart). Because of these possibilities that are morally concerning, and because these would not be pre-empted by the 14-day rule, as SHEEFs would always differ from human embryos just enough to fall outside the definition of a human embryo, Aach et. al. propose that guidelines should apply to SHEEFs' features as directly as possible. They suggest some features that could qualify as morally concerning (e.g. neurulation, occurrence of a heartbeat), the development of which may not be prevented by guidelines if they understand SHEEFs as entities that follow the conventional manner of embryogenesis. They emphasize on the fact that SHEEFs are entities whose development is plastic, one that can be modified and tuned in many ways due to new synthetic technologies. While SHEEFs' developmental capacities are cautiously qualified by these two teams of researchers, Pera et.al., provide commentaries that envision future research studies that may make efforts to culture SHEEFs with extra-embryonic cells from animals to create chimeric embryos (Pera et al. 2015). Such a direction of experiments, however, might become necessary if more needs to be found out about SHEEFs by culturing them beyond 14 days. But, SHEEFs becoming "embryos in a dish" and interfering with the 14-day rule in ways to suggest an inevitable modification or extension of the 14-day rule, is purely conjectural. Nevertheless, in this thesis, if not the most adverse of fates, the more

ambitious research projects involving SHEEFs are assumed for the purposes of offering an admonitory commentary.

Keeping such assumptions in mind, this thesis attempts to offer recommendations for conducting research on SHEEFs ethically. For instance, studying SHEEFs post appearance of a PS is encouraged to learn more about paracrine signaling, so that the emergence of different cell-lineages can be understood, on the more basic science side of this hESC technology. Understanding the embryonic stages that occur post 14 days is crucial to many fields of biotechnology (one such field being regenerative medicine), yet so little of it is understood and observed *in vitro*. Even though such studies may be difficult, given that correlation of SHEEFs to human embryos corresponding to those stages post 14 days would not be possible by dint of the 14-day rule, they provide a much better alternative to not having any such systems to study. However, efforts to implant SHEEFs (or hESC cultures that demonstrate a degree of likeness to human embryos) in non-human animals including primates would fall in the list of experiments that should not be conducted. Gestating or growing SHEEFs to term in any condition, be it in the uterus of an animal or in artificial wombs, would open a can of worms, presenting ethical dilemmas such as SHEEFs turning into animal-human chimeras, or SHEEFs becoming a human fetus[13], with the same genetic makeup as the parent cell of a SHEEF (which could be the donor of the hPSCs that formed the cell-lines SHEEFs were derived from).

[13] Again, this is purely speculative.

Given that the researchers who would conduct research on SHEEFs would determine the developmental stage SHEEFs are brought to, or the features that SHEEFs would exhibit, it is only appropriate for the guidelines to emphasize on researchers' intentions for culturing SHEEFs. As recommended by guidelines such as those formed by ISSCR, research projects involving hESCs or hESC culture models that have any organismal potential should be overseen by committees such as ESCROs (ISSCR 2016). Committees that oversee this type of research in an institution should seriously scrutinize the research projects, and follow through with the inspection of experiments, regularly, to ensure that the primary investigators are able to conduct the experiments as projected in their research proposal. The committees in charge of overseeing the research projects should also make sure that the research does not produce entities, production of which was not originally planned, or that the research is not continued even after the proposed experiment is completed. For instance, a research project that aims to create SHEEFs to study the process of neurulation (that occurs roughly 6 days after the appearance of a PS, in a typical embryogenesis), may not culture SHEEFs even after the neurulation stage, and should terminate the experiment and disintegrate the hESCs. These are just a few parameters that institutions charged with the responsibility to form guidelines on SHEEFs should keep in mind. As SHEEFs are, ultimately, hESCs that are being developed to certain organizational points in human embryogenesis, guidelines should take into notice the different morally concerning features that SHEEFs may develop and apply research limits accordingly. And, the examination of the merit of proposals of the primary investigator for research on SHEEFs should take precedence.

In Chapter 1, canonical embryogenesis and what it entails is explained so that comparisons can be drawn between human embryos and SHEEFs. The biological make-up of embryos will be discussed and how the growth of embryonic stem cells *in vitro* occurs will be described. In Chapter 2, piggybacking on the understanding of human embryos, it is noted that a separate classification for SHEEFs is required. In the same chapter, we also see how SHEEFs may be grown *in vitro* in future studies and how this could be regulated. In Chapter 3, laws, regulations, and guidelines pertaining to human embryos and hESCs are discussed in detail and some existing prohibitions on human-animal chimeras and human reproductive cloning are also discussed to note how implantation of SHEEFs in animals violates the same rules applied to chimeras or to clones. The 14-day rule and its emergence are also brought into light in order to note that SHEEFs need not be grouped into the same category as human embryos as the fundamental postulates that led to the 14-day rule do not apply to SHEEFs. And in the final chapter, I propose recommendations for SHEEFs' research and defend the arguments made with findings from the existing literature.

CHAPTER 1

In this chapter, definitions of human embryos and SHEEFs are given and their biological features are discussed in detail. In the introduction, it was shown that these terms can be confused often and while they share some similarities, they are different types of human cell-cultures that have different functions. Box 1, adapted from (Sapna Chhabra, Lizhong Liu, Ryan Goh, Aryeh Warmflash 2018), provides definitions of various terms that are important for understanding human embryos and SHEEFs.

Box 1: Definition of terms

Pluripotent Stem Cells: Cultured cells that may be grown indefinitely in the laboratory and can turn into any type of body cell. This includes embryonic stem cells, induced pluripotent stem cells, and other types of cells, such as epiblast stem cells, that correspond to early stages of development.

Multipotent Stem Cells: Multipotent cells can develop into more than one cell type but are more limited than pluripotent cells; adult stem cells and cord blood stem cells are considered multipotent (Department of Health, Wadsworth Center).

Totipotent Stem Cells: Totipotent cells can form all the cell types in a body, plus the extraembryonic or placental cells. Embryonic cells within the first couple of cell divisions (see the paragraph below the table) after fertilization are the only cells that are totipotent (*Ibid.)*.

In-Utero: Inside a uterus.

Artificial wombs: An artificial uterus (or artificial womb) is a hypothetical device that would allow for extracorporeal pregnancy (Bulletti et al. 2011) by growing a fetus outside the body of an organism that would normally carry the fetus to term.

Morphogenesis (or Embryogenesis): The process that leads to formational changes in the developing embryo, such as the development of organs and the spatial relationship of the tissues.

Gastrulation: A stage of development in which the primary germ layers (ectoderm, mesoderm and definitive endoderm), from which all the fetal tissues will develop, are formed.

Germ layers: The ectoderm, mesoderm and definitive endoderm cell layers that form at gastrulation, from which all fetal and some extra-embryonic tissues develop.

Patterning: The acquisition of localized morphological or molecular characteristics by cell populations in a tissue or an organ.

Primitive streak: A structure that forms in the posterior region of the embryo and is the first visible sign of gastrulation. Epiblast cells ingress through the primitive streak and are allocated to the mesoderm or definitive endoderm.

Epithelial-to-mesenchymal transition: A process during which cells lose their epithelial characteristics and gain an irregular appearance, lose their tight connection to each other, disperse and may become migratory.

Extra-embryonic tissues: Structures in the conceptus that arise from the zygote but do not contribute to the fetal tissues, including those derived from the trophoblast or from the primitive endoderm. These tissues support the growth of the embryo and are a source of signals for patterning.

Blastoids: Groups of trophoblast and embryonic stem cells that cooperate in vitro to form structures that morphologically and transcriptionally resemble embryonic day 3.5 blastocysts (Rivron et al. 2018).

i. DIFFERENCE BETWEEN TOTIPOTENT AND PLURIPOTENT CELLS

Totipotent cells, as described in the table, have the potential to become any and all cell types including embryonic cells and placental cells. Pluripotent cells, in contrast, can only give rise to embryonic cells but not placental cells. “Potency” of a cells is at its highest during the first few cell divisions, during when a cell is said to be totipotent.

As a cell divides and differentiates into several fixed lineages, it loses its potency and downgrades to pluripotent, multipotent, oligopotent, and lastly unipotent cells. Potency of a cell has been shown to be dependent on the cell's ability to activate certain genes located within itself. Induced pluripotent stem cell technology takes advantage of this very feature of a cell. After the 16-cell stage, the totipotent cells of the morula differentiate to become cells of either inner-cell mass of a blastocyst or the outer trophoblast and this stage is when the cells lose their totipotency (Wikipedia Contributors 2019). Cells of the human embryonic cell culture model created by Warmflash et al. were pluripotent and did not possess the capability to create cells of the placenta. As opposed to human cell culture models that are created from previously established embryonic stem cell-lines, human embryos *in vivo* have the quality to produce placental cells. In order to create human cell culture models that can survive for longer duration *in vitro*, and also engender placental cells, efforts could be made to grow SHEEFs with animal extra-embryonic tissues. Although this currently remains unknown, future experiments with SHEEFs of this nature can point towards some answers. The ethical ramifications of such experiments have to be explored as well, and this is discussed in the following chapters. To compare and contrast embryos with SHEEFs, in the next section, the biological makeup of embryos is looked at more closely.

ii. EMBRYO DEFINITION

Definitions of the 'embryo' vary. The Oxford English Dictionary (Oxford Living Dictionaries) defines an embryo as "the offspring of an animal before its birth (or its emergence from the egg)". In modern technical language restricted to 'the fœtus in utero before the third month of the pregnancy'(Maienschein 2014, 16). It is a stage from fertilization to the 8th week of pregnancy after which it is known as a fetus[14]. In this definition, it is clear that the embryo is not distinguished from a pre-embryo and the developmental processes that occur at this stage are assumed to flow together to form an adult, and hence, this definition may not be biologically precise. Some do not concur with this definition and discriminate between various stages. For example, the organism after fertilization and before implantation into the uterus is referred to as "conceptus" or "pre-implantation embryo" or "pre-embryo" because it is still in union with placental cells. Once it is liberated from the surrounding placental cells and forms a placenta that is separate from its self, although still attached with its host, the organism gets the title of an "embryo".

For the purposes of this thesis, I use the widely accepted, scientific definition of an embryo viz. "Embryo: An organism in the early stages of growth and differentiation, from fertilization to the beginning of the third month of pregnancy (in humans). After that point in time, an embryo is called a fetus. (Medicinenet.com)"

[14] "An unborn or unhatched offspring in the process of development, in particular a human offspring during the period from approximately the second to the eighth week after fertilization (after which it is usually termed a fetus)" – (Oxford Living Dictionaries)

iii. CANONICAL EMBRYOGENESIS

To understand how SHEEFs differ from human embryos and how their development resembles that of embryos, it is crucial to get a grasp on canonical embryogenesis, or division and differentiation of embryonic stem cells *in utero*.

In utero, after the fertilization of an ovum with a sperm occurs (on day 1), a number of events take place. The single fertilized egg splits into two cells within the next few hours and after that, a cascade of events results in the formation of 3 different germ layers: endoderm, mesoderm, and ectoderm, approximately, by the end of day 14 as noted in the Introduction.

The stages from the moment of conception (day 1) of the zygote to the formation of a fetus are:

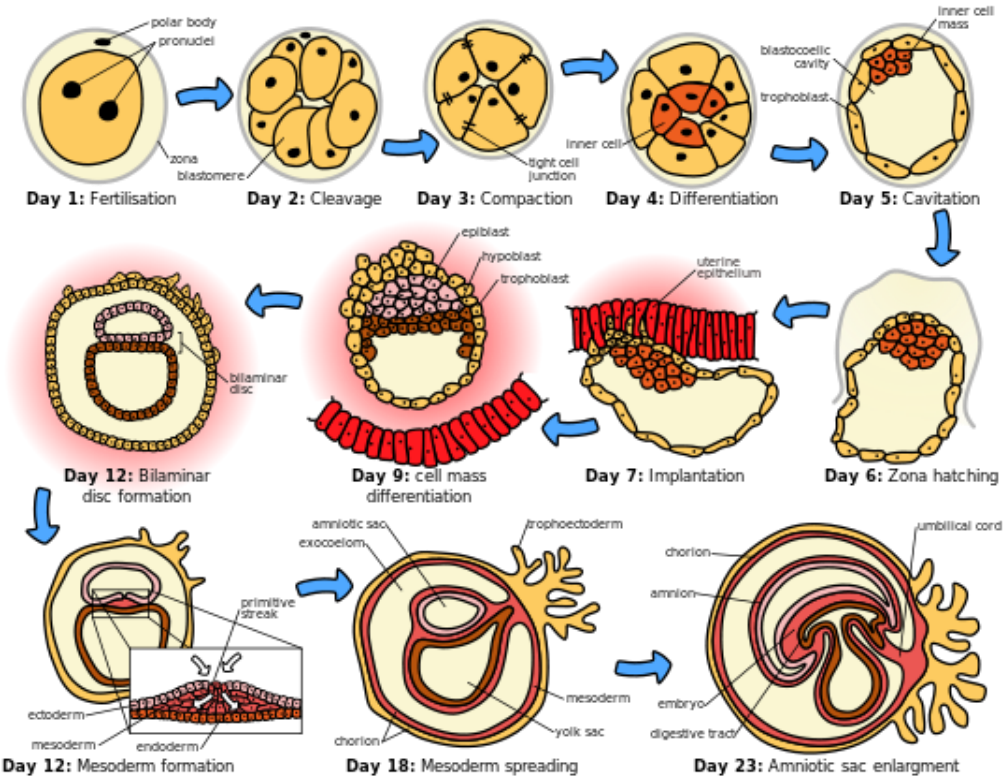
- cleavage,
- compaction,
- implantation,
- gastrulation,
- neurulation,
- mitotic cell division,
- regulated cell differentiation, and
- organogenesis.

Cleavage occurs between days 2 and 3, when one cell divides into two and those two cells divide into four and so on, exponentially, sharing the same volume inside a structure

called the zona pellucida. On day five, the number of cells reaches to a stage where the structure that is formed looks like a lump of cells. This is called the blastocyst stage – where division and differentiation occur, and stem cells are formed inside the **blastocyst**. This is called compaction, and on day eight, the blastocyst travels from the fallopian tube to the uterus to get implanted on the uterine wall which is being prepared for this moment. The blastocyst constantly receives signals that communicate commands about where to divide and, in the uterus, it further receives signals that propel the regulated differentiation of the cells. Now, the stem cells know which type of a cell they have to become[15]. The stage that occurs on the 14th day is called "**gastrulation**."

Cells inside a blastocyst are, primarily, grouped into three different layers in the gastrulation stage: ectoderm (outermost), mesoderm (intermediate), and endoderm (innermost). Cells in the ectoderm differentiate into cell lineages that form hair, neurons, skin, etc., cells in the mesoderm form heart, muscles, bone, blood, etc., and cells in the endoderm form intestinal lining, bladder, pancreas, etc. By 8 weeks of gestation, i.e., the first trimester, the embryo begins to develop most organs and body parts – eyes move to the front, limbs take a definite shape, has cardiac cells, etc. and at that stage and forward, it is called a fetus.

[15] Some stem cells are preserved in some parts of the body and they perform activities such as replacing damaged cells throughout a lifetime.



"Human Embryogenesis *in vivo"* - Picture taken from (Wikimedia Commons Contributors and Zephyris 2018)

After two months, the embryo has begun to form all major organs and body structures. At 12 weeks, which is also called the first trimester, the embryo is considered to have become a fetus. The fetus, now, has an audible heartbeat. The fetus continues to grow inside the uterus following a natural conception (or via assisted reproductive techniques) to differentiate further and develop all organs of the body.

At the blastocyst stage, it is possible to extract one or more cells and use them as a source of Embryonic Stem Cells (ESCs). ESCs are totipotent and can differentiate into varies types of cells in the human body (neuronal, muscular, etc.) Since the discovery of Induced

Pluripotent Cells (iPSCs), ESCs are not used as much as they were before to grow into different cells and tissues for scientific research (de Wert and Mummery 2003). But research continues on embryos that are a by-product of IVF for reproductive purposes and hESC-lines continue to be derived from pre-implantation embryos, including embryos created for research purposes and embryos left over from IVF.

Research is conducted on pre-implantation embryos (and leftover IVF embryos) to understand many genetically inherited diseases (e.g., mitochondrial diseases among other chromosomal aberrations), to study the development of the organ systems from different germ layers, to understand neurodegenerative diseases, to derive ESCs, and to understand fertility, among other things. In the next section, the development of ESCs in laboratory conditions is looked at more closely to understand how SHEEFs are formed.

iv. ROLE OF EXTRA-EMBRYONIC SIGNALLING IN AN EMBRYO'S GROWTH

ESCs are usually derived from existing cell lines or from embryos donated for research. These cells, due to their divisional capabilities, have multiple uses in biomedical technologies. ESCs are used to regrow damaged tissues in patients, i.e., in regenerative medicine. ESCs are genetically modified and introduced in mice to observe their division and growth. A lot has been learned about embryonic development and disease from creating 'chimera' mice with genetically modified ESCs to test how specific genes contribute to disease and function (Embryonic Stem Cell Factsheets). A major focus of

developmental biology is to observe the growth of ESCs *in vitro*. Studies to discover which signals differentiate stem cells into specialized cells are undertaken, as well. Researchers are also learning how to assemble complex tissues to form layers of the brain and grow primitive organs in the lab. The challenge is to understand the crosstalk between these signals and cells *in vivo* which can be exploited to grow ESCs in the lab. Since the first few weeks of embryonic development in utero is difficult to monitor, this early period is known as the black box of developmental biology, which is why researchers try to grow these cells in vitro instead, to understand canonical embryogenesis better. Observing embryonic development in lab gives us an insight into how cells arrange themselves inside a uterus. Let's take a closer look at the signals that dictate the embryo's growth.

Cells and tissues inside our body obtain signals from other cells and tissues, which allows them to spatially grow and divide, as noted in the previous section. Try to replicate the same on a dish and you wouldn't expect the same. Cells grow in two dimensions *in vitro*. In dish conditions, cells (and for our discussions embryonic stem cells) get signals from neighboring cells but more strongly from the growth medium[16]. When human embryos were observed *in vitro*, spatial differentiation, i.e., embryonic cells' splitting in three dimensions and consequently into three different layers - was observed, and this theory was used to propose that the same could be mimicked with ESCs *in vitro* to obtain perfectly differentiated germ layers (Berge et al. 2008; S. C. van den Brink et al. 2014; Harrison et al. 2017). Other examples are: (Morgani et al. 2018), (Tewary et al. 2017). In these studies, controlled growth of the stem cells – "geometrical confinement", or "spatial confinement",

[16] The cell-culture medium that the cells grow in. It usually consists of nutrients, different biological markers, and growth media like fetal bovine serum. (Growth medium 2019)

or “micropatterning” - resulted in the formation of the three germ layers in the center of the colonies of the cells.

Inside a uterus, after gastrulation, a human embryo receives information from the surrounding maternal cells (these are called extraembryonic cells), such as visceral endoderm and trophectoderm (Arnold and Robertson 2009). Development of an embryo occurs spatially and is coordinated by various signals. These signals can be understood as a commander that gives out very specific instructions to the cells. In the case of cell differentiation, such as in gastrulation, these instructions are mostly positional, in that the instructions primarily direct the cells to proliferate in a designated location.

A BMP[17] signal, *in utero*, commands cells to develop from the center toward the circumference (Siggia and Warmflash 2018). The studies mentioned above mimicked this in-vitro by using a marker called BMP4. BMP4 treatment mimics the BMP signals from the trophectoderm[18] (trophoblast tissues), causing hESCs (derived from established cell-line cultures) to become more extraembryonic or epiblast-like. These BMP signals further activate Wnt signals, which are inhibitors of BMP. As in every biochemical reaction, a signal can be an agonist – directing the cells to do task A, or an antagonist – directing the cells not to do task A. BMP can be understood as an agonist with Wnt as its inhibitor. The activation of BMP and Wnt cues the activation of Nodal. As BMP prompts the cells to divide and proliferate, Wnt and Nodal prompt them to stay within a boundary (here, a

[17] BMP4 is the in-vitro cell signaling counterpart of BMP (bone morphogenetic proteins).
[18] “[Trophectoderm is] a layer of tissue on the outside of a mammalian blastula, supplying the embryo with nourishment and later forming the major part of the placenta.” (Wikipedia contributors 2018)

sphere). BMP signals direct the position of mesodermal differentiation and Wnt and Nodal signals maximize differentiation far from the mesoderm (Sapna Chhabra, Lizhong Liu, Ryan Goh, Aryeh Warmflash 2018). Due to these signals and the cells dividing and segregating into different layers while simultaneously taking their place in a spatial geometry, a structure that resembles a blastocyst with an inner cell mass is developed.

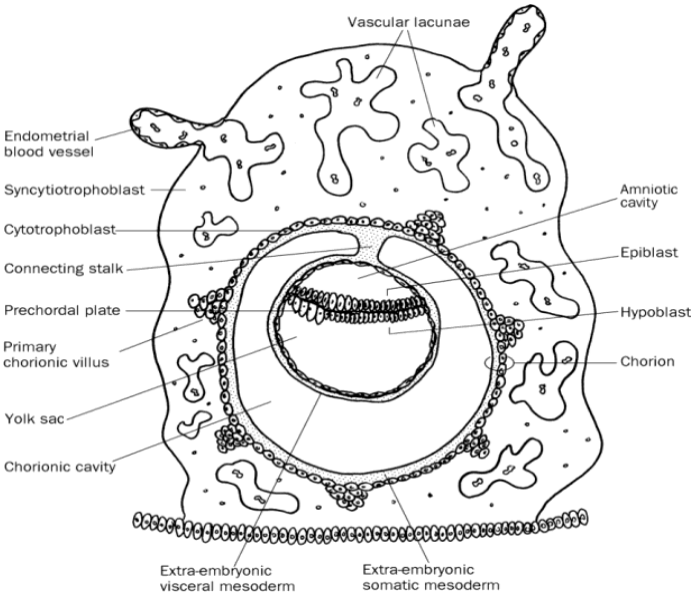


Figure showing Blastocyst and Structure of Placenta on the 14th day taken from- https://www.med.umich.edu/lrc/coursepages/m1/embryology/embryo/04secondweek.htm (University of Michigan Medical School 2000)

This is how the inner cell mass of a human embryo looks when observed under microscope on the 14th day (see figure), concentrated in a spherical shape inside the yolk sac.

Together, BMP, Wnt, and Nodal signals regulate gastrulation at the proximal end of the embryo. The primitive streak (PS), described earlier, is the site of gastrulation where the three germ layers (endoderm, mesoderm, and ectoderm) begin to develop. The spotting of a PS is therefore a sign of the beginning of gastrulation. The spotting of a PS is also indicative of the fact that further differentiation of the cell into two different embryos (twinning) would not occur, making it a stage where the biological individuality of an embryo is attained.

Much of the understanding about the roles these signals play came from studying mouse embryos. A prominent study that contributed to this field was recently performed by Rivron and his team at MERLN (Institute for Technology-Inspired Regenerative Medicine). They experimented with mouse embryonic stem cells by subjecting them to trophoblastic cells (N. C. Rivron, Frias-Aldeguer, Vrij, Boisset, Korving, Vivie, et al. 2018). This and other studies later done to understand self-organization within embryonic cells *in vitro* helped in understanding the early stages of an embryo (Bedzhov and Zernicka-Goetz 2014; S. C. van den Brink et al. 2014; Harrison et al. 2017; Shao et al. 2017; Warmflash et al. 2014a). It was seen in the Rivron study that when the mouse ESCs came in close proximity to the trophoblastic cells (amniotic cells) they started dividing and exhibiting characteristics akin to that of embryos. We see in the next section how this was used to create SHEEFs by Warmflash and colleagues.

CHAPTER 2

i. SHEEFS – TERMS AND DEFINITIONS

As new ways to create ESCs and PSCs in lab are explored, an effort to create human cell-culture models to be used as alternatives to human embryos for research has been under way. As introduced in the previous section, Warmflash and his team created synthetic human entities in laboratory by using hESCs and subjecting them to BMP4 and other extra-embryonic[19] markers and geometrically regulating their cell division and multiplication[20] through micropatterning.

These human cell-culture models were called **Synthetic Human Entities with Embryo-like Features,** or SHEEFs, by a team from Harvard (Aach et al). They are not equivalent to human embryos (Rivron et al., 2018). Human cell-culture models mimicking early human development may be identified according to the stage that they are intended to mimic or the stage that the investigators want to study. The term "gastruloid", as was noted in the introduction, is used to describe a human cell-culture model designed in the lab to study the stage of gastrulation, i.e., splitting of cells into three different layers –

[19] Also known as trophoblast signals because trophoblast is the adjoining tissue of a uterus in which the embryo is implanted. Also, could be understood as the layer in contact with the epiblast – or outermost layer of an embryo. These signals are also called "morphogens" as they bring about morphogenesis or "change".

[20] See this video demonstrating in-vitro culturing of hESCs in Warmflash et al's lab where the cells show a growth and differentiation pattern similar to a human embryo culture in-vitro. (Artificial Human Embryos Are Coming, and No One Knows How to Handle Them 2017)

endoderm, mesoderm, and ectoderm. A “blastoid” is a term that is used to study the blastocyst stage of an embryo. In his paper, author Nicholas Rivron uses “blastoid” as a replacement for “synthetic blastocyst” (N. Rivron 2018), the stage of human embryo that occurs at roughly 3.5 days after fertilization. Therefore, calling the entities in Warmflash and colleagues' paper “embryoids” should also serve the same purpose. But by doing so there is a risk of imparting a message that these are human embryos and thus might have whatever moral status or warrant whatever respect one accords to human embryos. The term “embryoid” may risk portraying these entities as markedly similar to human embryos when going by the current research, that may not be so.

In my opinion, SHEEFs is the best term to describe these entities, as synthetic entities that have embryo-like features because it clarifies that these entities are “embryo-like” and that they are “synthetic”, i.e., created by man. As this technology to grow hESCs through micropatterning continues to develop and become more refined, there may be a need to clarify whether these hESC colonies need to be given a higher moral standing than other forms of human cell culture models. The existing research on SHEEFs does not indicate that SHEEFs be implanted into humans or animals, in which case, them materializing into persons may never occur. SHEEFs in Warmflash and colleagues’ experiments did not warrant any moral consideration; and I discuss more about why this is in the following sections. Therefore, SHEEFs may remain significantly different from human embryos[21] to exempt them from rules that apply to embryos. But, as they develop

[21] Produced via IVF using gametes derived from humans, through artificial insemination, or by sexual intercourse.

further, there may be a need to assess the moral significance of the biological features they exhibit. (See further discussion on "potentiality" in this chapter).

Using the term SHEEFs instead of micropatterned hESC colonies (as it was used by Warmflash et al), in my opinion, will slightly broaden the definition to cover entities that, in future, other laboratories may create out of induced pluripotent stem cells[22]. Henceforth, the term SHEEFs as used in this thesis will refer to the human cell-culture models that are created in-vitro to recapitulate early human development[23].

ii. SHEEFS AS GROWN IN WARMFLASH AND TEAM'S LAB

In Warmflash and Brivanlou's paper of 2014, they explain the interplay of SOX2, NANOG, BRA, and OCT4 signaling. Their paper concludes that SOX2 is observed in high levels in SHEEFs in the center, and NANOG and OCT4 are observed in higher levels toward the epidermis (suppressing mesodermal growth). The expression of these signals, especially BRA (brachyury – which is considered the signal that contributes the most towards extra-embryonic growth), which is pivotal in forming ectoderm, leads to "the emergence of all three germ layers in the micropatterned cultures [suggesting] that cells might be patterned by gastrulation-like events in a region resembling the primitive streak." (Page 849, Warmflash et al. 2014). Due to this very near recapitulation of human

[22] Adult cells that are reprogrammed to their pluripotent stage. Induced Pluripotent Stem Cells (iPSCs) could be taken from patients to develop their human embryonic models to understand their particular disease more in depth.
[23] And this is not restricted to 15 days post fertilization, early human development also includes the fetal stage which occurs much later.

embryogenesis *ex utero* the hESCs grown by Warmflash et al. were fated to become more embryo-like than other human cell-culture models of embryonic development that were created before these in their previous papers. In their own words, "[T]hus, cells grown on patterned substrates are a sensible approximation to the early gastrula and more appropriate than a solid embryoid body." (Page 852, Warmflash et al. 2014)

In their paper, Warmflash and his team also state the importance of micropatterning in understanding the underlying mechanism of how these extra-embryonic signals communicate with hESCs, and this may help to produce spatially ordered tissues for clinical purposes. They also mention the use of micropatterning in comparison studies between iPSCs and hESCs, in similar assay conditions, concluding that geometrically controlled cell-cultures should become the standard practice for ESC differentiation (Warmflash et al. 2016).

A few similarities between SHEEFs and human embryos were noted in the previous chapter. However, there are significant differences that are hard to find in the existing literature that have been expounded in another paper by Warmflash and colleagues (Sapna Chhabra, Lizhong Liu, Ryan Goh, Aryeh Warmflash 2018). One of the primary differences is in the formation of the PS. In a human pre-implantation embryo, the PS forms at the caudal midline of the flattened disk, whereas in SHEEFs, it is formed in a continuous and circular fashion around the structure. Also, the radial symmetry (i.e. rostral – caudal or anterior – posterior) was not mimicked in SHEEFs. The third and the most important difference between the two structures is that SHEEFs only expressed a few genes of the

many in human embryos *in vivo*. Martin Pera, in his article, stresses the importance of this difference by adding that the markers used to test for these genes were markers that are representative of germ layers of mouse embryos and not of human embryos, as there are no data from the latter (Pera et al. 2015). In other words, not enough biological markers to identify genes belonging to human germ layers have been discovered due to the limitations on studying implanted embryos. For their experiments, Warmflash and colleagues, like other developmental biologists, were required to gather the knowledge about the interplay of signals by studying animals[24], and the same is also true about the screening for germ layer cells' genes. And this makes validating human genes, and in turn testing the similarities of germ layer tissues of SHEEFs to human germ layer tissues, difficult. Aach et. al. mention some more factors that limit hESCs from following a growth pattern similar to human embryos; one such factor being the "effective means for delivering nutrients to and eliminating wastes from cells in the organoid interior." (Aach et al. 2017a, 4)

Being able to establish similarities and differences between SHEEFs and human embryos requires a thorough understanding of a human embryo. This includes studying pre- and post-implantation embryos, as there is no other means to identify the morphogens supplied to the embryo by the uterus. This obstacle could be overcome by co-culturing SHEEFs with extra-embryonic tissues. As briefly mentioned in the introduction, advances in extracorporeal pregnancy could also revolutionize this category of developmental biology. However, future studies of SHEEFs may not be able to attain full resemblance (genetically and anatomically) to a human embryo without knowing more about human embryos, which

[24] "Studies in fish, frog and mouse embryos have established that spatial patterning during gastrulation is under the control of the Activin-Nodal, BMP and WNT pathways." (Page 847, Warmflash at al., 2016)

would require studying them post 14 days. Some authors have suggested the extension of the 14-day rule to accommodate future studies on human embryos for a longer period (Appleby and Bredenoord 2018). Both solutions to the problem of facilitating further research on SHEEFs are not without legal and ethical limitations. A detailed proposal of ways to facilitate research on SHEEFs in order for them to replicate human embryogenesis is outside the scope of this thesis.

iii. IMPLANTATION VS. DESTRUCTION

SHEEFs are models of human cell-cultures created in order to study early human development. Early human development ranges from the moment of fertilization to the neonatal stage, and while most laboratories do not study embryos beyond 14 days for ethical or legal reasons and instead destroy or freeze them at that point, it may be possible to study SHEEFs for much longer than that. This is due to the fact that SHEEFs currently do not fall under the human embryo category and instead belong to the human embryonic stem cell category and are not regarded to be as intact as human embryos (Aach et al. 2017a). This implies that SHEEFs may be allowed to develop beyond 14 days in the future even though Warmflash and colleagues terminated the experiment and destroyed them after 15 days of culturing due to the reasons mentioned in the introduction[25]. Moreover, the artificial nature of SHEEFs makes it possible for the experimenters to manipulate the micropatterning conditions to help make the appearance of a PS and the gastrulation stage occur earlier than 14 days post fertilization.

[25] SHEEFs were destroyed by Warmflash et al to abide by the 14-day rule as the embryonic stem cells (ESCs) that were used to culture the gastruloids were of human origin and were able to mimic a human embryo at that stage.

Should SHEEFs be studied even after the appearance of a primitive streak, whenever it may be? As we see more in chapter 3, the 14-day rule presents reasons to regard the appearance of a PS as an ethical and agreed upon caution, but should this be so regardless of the entity it is observed in? Assuming that SHEEFs are exempted from this rule, should they be studied indefinitely? Or should there be a new limitation somewhere further down the line of embryogenesis? And taking a huge leap of faith, assuming SHEEFs are exempt from the 14-day rule and in later experiments, if SHEEFs are allowed to be cultured to term, should attempts be made to implant SHEEFs? These assumptions are based on the biggest, and most crucial of them all, assumption that SHEEFs *can* be cultured successfully beyond 14 days, and some day in the future, to term.

Culturing SHEEFs for longer durations and studying their growth should be promoted provided that the experiments undergo necessary scrutiny and comply with guidelines that will be prepared for SHEEFs in the future. Experiments with research questions that are designed to use SHEEFs for regenerative medicine should be promoted and these experiments may also take advantage of developing SHEEFs at a faster pace than canonical embryogenesis[26]. And for reasons like these the plasticity and reproducibility of SHEEFs, for instance, by forming a PS or developing into certain cell-lineages faster, or producing several copies of SHEEFs, might prove to be advantageous. However, as

[26] This is purely speculative. In current research on SHEEFs, there is no mention of SHEEFs or other human embryonic stem cell culture models having the capacity to develop faster than typical embryogenesis process.

the potentiality of SHEEFs to develop neurons capable of perceiving pain or responding to stimuli is not known, any experiments that aim to create SHEEFs and study them beyond 14 days should undergo thoughtful scrutiny. Professional guidelines in some countries state that as long as ES cell cultures are not intact and can be disintegrated after research, culturing them in laboratory will not cross paths with regulations on embryos (Pera et al. 2015)[27]. We will see, in the third chapter, that there are guidelines currently on research using human cell culture models that have organismal potential that require EMRO supervision.

In this section, we will look more closely at these questions and I propose my first argument that SHEEF research should be subject to rules and guidelines separate from those that apply to embryo research. And in the third chapter, I will further discuss the possibility of implantation of SHEEFs in human/artificial wombs creating reproductive clones, and in animals, creating human-animal chimeras.

iv. NEED FOR SEPARATE GUIDELINES FOR SHEEF RESEARCH

While they may lack the cellular progenitors to instruct the stem cells to differentiate into different lineages like heart, placenta, brain, or plasma, these cells that make up SHEEFs divided and differentiated like human embryos up till 14 days in vitro (Warmflash et al.

[27] "…It should, however, be noted that in those countries that permit the generation of new human ES cell lines, it is generally considered acceptable to maintain cultures of embryonic cells beyond 14 days as long as the embryo is disaggregated or otherwise not maintained intact." (Pera et al., 2015)

2014a). Aryeh Warmflash, whose lab is currently researching micropatterning[28] in ESCs, works toward answering developmental biology related questions using mathematical modelling in cell-fates. Given the ethical and technical restrictions on studying human embryos beyond 14 days or the appearance of the PS, Warmflash and his team say on their website that the creation of these embryoids, which they call "hESC system", is a step toward understanding what comes after 14 days.

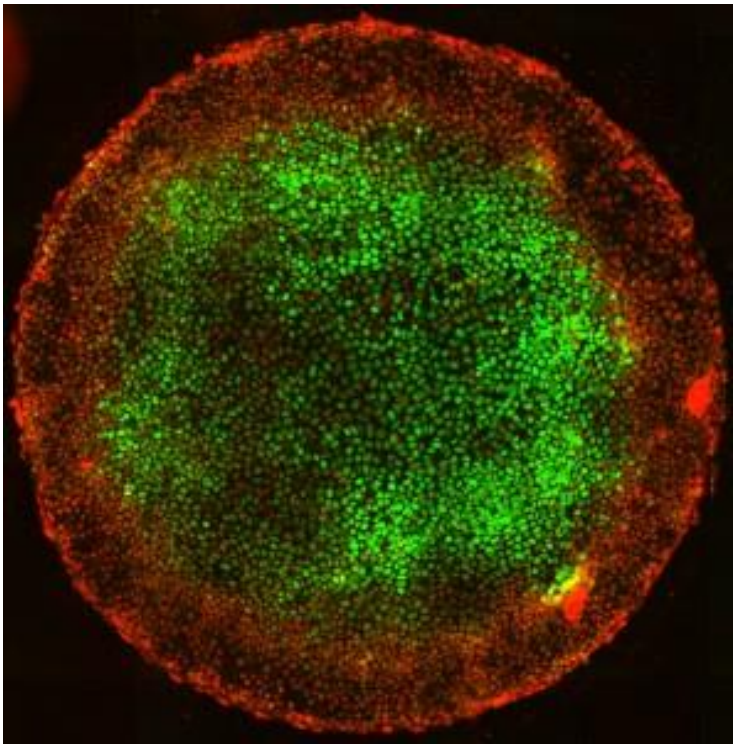


Image: "Patterns of cell differentiation" taken from https://stemcell.rice.edu/research/ (Laboratory of Systems Stem Cell and Developmental Biology)

[28] A paper published in 2016 titles "Self-organization of the human embryo in the absence of maternal tissues." B Shahbazi *et al* also shows that creating embryoids in-vitro with pluripotent stem cells was possible. (Shahbazi et al. 2016b) But, unlike the Warmflash et al paper these cells did not exhibit later epiblastic features in the absence of micropatterning.

The appearance of a PS can be understood as a precursor event of development of the central nervous system (CNS) that aligns the cells' development from head to tail. In a typical process of embryogenesis or "canonical embryogenesis" after the appearance of PS, approximately on the 14th day, the process of "neurulation" occurs and simultaneously, depending upon the cell signals, cells form the three germ layers (endoderm, mesoderm, and ectoderm), which develop into their respective tissues and organs[29]. The hESCs, in Warmflash et al, that were treated with BMP4 and manifested a PS, mimicked the canonical embryogenesis of a mammalian embryo until the "gastrulation" stage. If the SHEEFs had been allowed to develop beyond 14 days, it would have been possible to see if they mimic[30] further stages of embryonic development such as the neurula stage (occurring at ~28 days)[31]. Attempts may also be made to use SHEEFs for implantation[32] in artificial wombs or animals as noted in previous sections. The current research, however, is far from observing more mature stages of embryonic development such as the formation of cardiac cells or reaching the neurulation stage. Since research on SHEEFs is at rudimentary stages, one must also bear in mind that SHEEFs created in Warmflash and team's laboratory were not totipotent (i.e. capable of developing into later stages of human embryos)[33] (Appleby and Bredenoord 2018).

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[29] The cells in the
- i) ectoderm form epidermis or skin cells,
- ii) mesoderm form internal organs like heart, kidney, skeleton, red blood cells and smooth muscle cells, and
- iii) endoderm form lungs, pancreas, and thyroid glands

(Germ layer 2019)

[30] This may not be the definite outcome.

[31] Development of SHEEFs to such a stage in embryogenesis requires that scientists understand what conditions to subject SHEEFs to in order to get to that stage. This, however, is not possible without understanding human embryos. Assumptions based on the understanding of mouse embryonic models can be made to develop SHEEFs beyond 14 days, but to what extent this can result in SHEEFs that can aid in transplantation in humans is not known.

[32] In humans and animals.

[33] By themselves.

Nevertheless, it remains a constant quest for developmental biologists to create human cell-culture models to study these features.

A recurring theme that will be observed in this thesis is that given the differences between the two entities, human embryos and SHEEFs, they should not even be categorized in the same group for the purposes of regulating research. Should a structure that could resemble an embryo in this manner, although not formed out of gametes, be treated as if it were an embryo and be subjected to the same research rules? Or, because they are derived from human embryonic stem cells, should they be subjected to the same research rules as hESCs? SHEEFs should not be put in the embryo category, nor should they be treated merely as hESCs. SHEEFs need a sub-category under human embryonic stem cells and they should also be researched on further to be used as suitable replacements for animal models and to avoid embryo-destructive research. SHEEFs, I argue, need their own category under hESCs because they are comprised of pluripotent hESCs and their configuration into and development as SHEEFs depends entirely on the how researchers micropattern SHEEFs. Thus, for regulating SHEEFs, the key consideration for research oversight is the researchers' intent and design.

v. PUBLIC POLICING OF SHEEFS RESEARCH SIMILAR TO THE 14-DAY RULE BUT NOT THE SAME

Embryo destructive research, although a common scientific and ethically permitted practice in some jurisdictions as long as the 14-day rule is upheld, is still condemned by many organizations and individuals[34]. The 14-day rule receives criticism from groups of people who want to extend it to accommodate more research, and from groups who oppose all kinds of research on embryos. Some groups of people also believe that extending the rule may undermine trust in research by not honoring the agreements that were made regarding embryo research. Proposals to extend the 14-day rule are also met with criticisms because some people regard it as a bright moral line specifying when the human embryo obtains a moral value. But the 14-day rule should be understood as a public policy tool, formed after decades of international and national conferences, after reviewing recommendations submitted by scientists and bioethicists all over the world, to strike a practical balance between enabling research and maintaining public trust (Hyun, Wilkerson, and Johnston 2016). Similarly, for the development of a set of guidelines for SHEEFs, a public policy tool such as a 14-day rule should be considered. And since public policy tools are modified as scientists discover more efficient ways for creating human cell-culture models for early embryonic development, SHEEFs will also undergo modifications as studying them for longer durations becomes a possibility. Meanwhile, it is incumbent upon research institutions such as Stem Cell Research Oversight committees (SCRO)[35] to come up with initial guidelines on how SHEEFs may be used for research. I propose that SHEEFs can be placed in the human embryonic stem cell category while considering the formation of international guidelines. What these guidelines may look like, is discussed in the following sections.

[34] For example, see: ("The Case Against Humanity", MCCL.org)
[35] And others mentioned in the introduction.

While placing SHEEFs under human cells and tissues accords with their biological nature, depending on how they are used in an experiment their capacity to develop enough to perform functions like perceiving pain or breathing could raise some ethical questions. Therefore, it would be ideal to assess the usage of SHEEFs depending upon the experiments. For example: an experiment designed to study the gastrulation stage, villi formation, early placentation, and folding of the embryonic disk, which occurs at 15 days post fertilization, commonly referred to as Carnegie stage 7[36], could be understood as an ethically permissible experiment insofar as observing these events in vitro is possible. In contrast, an experiment that is designed to observe the stage equivalent of a 38-week human embryo may need serious forethought. At 38 weeks, a typical human embryo in vivo[37] has a well-defined system of blood vessels, cranial nerves, spinal cord, and more internal features[38]. It externally presents features that are all indicative of a mature fetal structure. And experiments designed to observe SHEEFs at a stage equivalent to this in vitro should be given more consideration – assuming SHEEFs can be studied up to this point, should they be?

Earlier we talked about human cell-culture models being given different names depending on the embryogenesis stage they are created to mimic. If SHEEFs are created to study only a particular stage in human embryogenesis, they would no longer be understood as equivalent to a human embryo intended for continuous and integrated

[36] For more information, see – under week 3 (Hill).
[37] One that is developing in a womb as studied in the 19th and early 20th century (Mall, 1891)
[38] See external body form by (Barniville 2018)

development toward live birth[39]. This, in addition to the reasons stated in paragraphs above, I posit that SHEEFs and embryos do not belong to the same category. Therefore, it follows that they be subjected to different regulations and research on them may follow different guidelines; this should include their exemption from the 14-day rule. In fact, any proscriptive rule such as the 14-day rule may fail to keep up with the advancement of human cell culture models (Hyun and Wilkerson 2016). Committees that oversee scientific and ethical compliance of research studies with international guidelines may be more apposite. Guidelines should be taking note of each case; gauging the risk and benefit of the research involving SHEEFs. Research on learning more about developmental science should be encouraged over using micropatterned SHEEFs to be implanted into animals. More about rules, regulations, and guidelines is discussed in the next chapter, with this section only recommending the need for a separate category.

vi. POTENTIALITY

Embryos created through IVF have the potential to develop into a person after a successful implantation and incubation to term. ESCs do not possess this potentiality, but cultured through micropatterning, these human cell culture models are able to mimic the process of standard embryonic growth to an extent. Does this grant these human cell culture models (SHEEFs) potentiality to develop to term? Given the current research this may not be so easy to conclude, but can future efforts to grow them for longer durations change this?

[39] This argument was also presented in (Pera 2017)

Research on artificial wombs and co-culturing SHEEFs with extra-embryonic animal tissues could show promise for providing a growth environment for SHEEFs. This could enable SHEEFs to attain more advanced stages of embryogenesis and trigger the use of SHEEFs for reproductive purposes. Implantation of SHEEFs in humans could be a shocking advance and scientifically far-fetched. But will their implantation in animals or their growth with extra-embryonic tissues elicit the same shock, or be equally technically challenging? This should be given serious consideration before research in that area is allowed to progress.

The ramifications of implanting SHEEFs or creating different types of SHEEFs from existing cell-lines are that they pose ethical dilemmas similar to those posed by reproductive cloning. Co-culturing SHEEFs with animal extra-embryonic tissues, say, mouse extra-embryonic tissues because of the ease of studying mouse-models, also presents ethical issues akin to those presented by animal-human chimeras(Kurtz and Oh 2016)(Wu et al. 2017). Creating animal-human chimeras by using human cells to create organs in animals, or by attempting to grow a human stem cell admixture in animals, could cause for human cells to end up in the brain of the chimeric animal, giving raise to mental cognition and this could have unanticipated effects; the harm being the exploitation of animals for research. Whether the type of the chimeric structure formed out of the mixture of hESCs and animal tissues is classified as animal-human chimera, or human, or animal, is a bioethical question; one that may have different answers depending on the exact methodology of the experiment. More on this is discussed in the

next chapter. Nevertheless, in order to maintain public trust in science and in this niche of developmental biology, it is crucial to give considerable thought to these research questions and observe a hiatus before exploring them.

Efforts to maintain SHEEFs for longer durations, or to co-culture them with extra-embryonic tissues, or to implant them into animals, are still very technically challenging due to limitations of the equipment, or due to the lack of knowledge of culturing human tissues beyond 14 days. The first step for research on SHEEFs to progress in this direction would have to be the approval of studies that aim to culture SHEEFs beyond 14 days. Assuming that is the case, and that SHEEFs can be studied and grown with extra-embryonic tissues, it would be crucial to determine if research on SHEEFs that intends to use them for more than basic developmental biology, or for producing tissues for clinical purposes, should be performed. This poses yet another challenge pertaining to the potentiality of SHEEFs as with their co-culturing with extra-embryonic tissues they begin to develop features that may require more consideration than a PS.

It could be argued that the development of neural tissues is a necessary criterion for sentience; going by that SHEEFs, at a stage later in the development, could possess a higher moral status than SHEEFs at 14-days. It could be argued that SHEEFs created for the purposes of therapeutic cloning or to create cells and tissues for transplants, possess at least the degree of potentiality that embryos discarded in fertility clinics do. Moreover, for SHEEFs to attain individuality they must possess a unique genetic makeup (unless it is the case of biological twins), the purpose of which is defeated when SHEEFs are

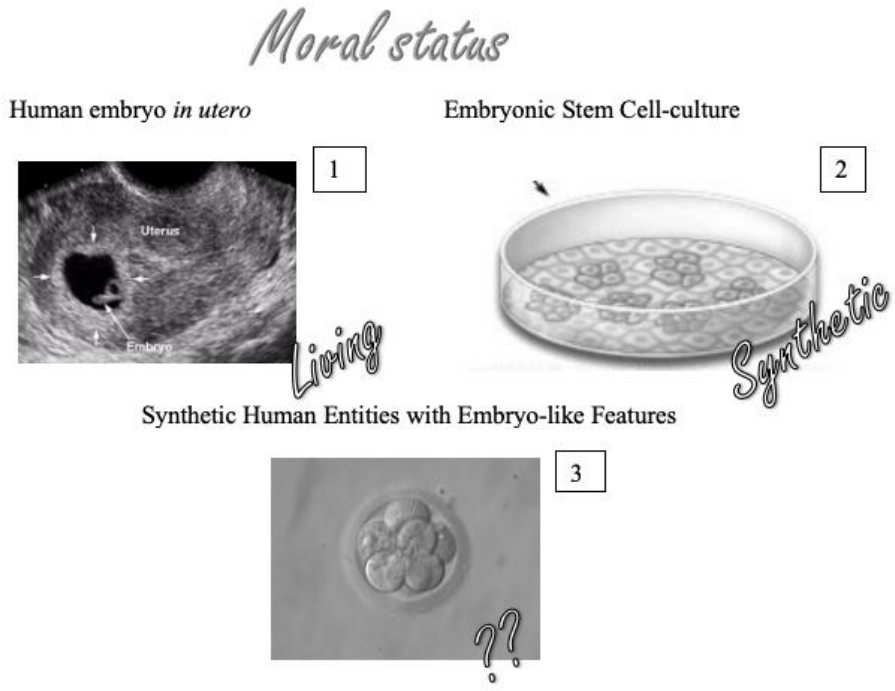
cultured from pre-existing cell-lines or from iPSCs of patients. SHEEFs originate from previously established cell-lines or embryos donated to research. The genetic makeup of a single SHEEF will be identical to its donor's. This not only involves consent issues for implanting a SHEEF in a human or an animal (in a hypothetical, futuristic experiment) but will also risk possibility of cloning the donor. And, this also applies to SHEEFs created via therapeutic cloning (SCNT or PSC induction) as the genetic identity is passed down via the input nucleus in SCNT or passed down to the stem cells through donor's DNA.

We see in the next chapter that the creation of reproductive clones or chimeras are subject to some laws in every country and creation of SHEEFs may also violate these existing laws insofar as they are considered to be human derived stem cells (or other types of human tissues that these laws were formed for).

As mentioned before, creation of these advanced forms of entities is not currently possible and efforts to study SHEEFs beyond 14 days should be made, and research institutions like ESCROs should evaluate the merit of experiments. SHEEFs should be studied beyond 14 days to learn more about their potential and to use them in regenerative medicine to create organs by using a patient's iPSCs. However, experiments using SHEEFs to create chimeras or to implant them into animals should undergo graver scrutiny[40]. Therefore, an implicit theme observed in this thesis once again presents itself

[40] Sometimes, growing artificial organs is only possible by implanting human cells in animals, and this collides with the issue of chimeras. Discussion on this possibility is vital but is out of the scope of this thesis. In my opinion, when it becomes imperative to use hESCs in animals to grow organs, it leads to a slippery slope, and alternatives to this should be researched on.

to us – the duration SHEEFs are cultured for, and the capacity to which SHEEFs are created to be used by researchers, together determine the profundity of oversight. This is due to the development of features such as neurulation, vascularization, observing a heartbeat, that may warrant more attention which changes depending on how SHEEFs are cultured. For instance, SHEEFs grown to reach a stage equivalent to a 28 day embryo may be micropatterned to reach the stage of neurulation, and this could warrant more attention as at this stage neurons could be observed, indicating response to stimuli and basic pain perception.



Sources of images used

1. (Doubilet and Goldberg 2013)
2. (Heins et al. 2004)
3. (Howe 2017)

Based on the arguments provided in this chapter, I propose that a separate category for SHEEFs under human embryonic stem cells should be considered.

CHAPTER 3

In this chapter, existing laws and guidelines on human embryos, on chimeras, and on reproductive cloning are looked at closely. In the second section of this chapter, the background of the 14-day rule is discussed. Arguments made in previous chapters about SHEEFs' potentiality and their uses as replacements of animal models and human embryo-destructive research or their uses in regenerative medicine are discussed further. In this chapter, I give reasons why human-animal chimera research projects involving the usage of SHEEFs to be co-cultured with animal extra-embryonic tissues, or implantation of SHEEFs in humans or animals, should not be undertaken until the establishment of guidelines on SHEEFs.

i. LAWS AND GUIDELINES THAT REGULATE RESEARCH ON HUMAN EMBRYOS

In the early 2000s, the Center for Disease Control ("CDC") came up with regulations for fertility clinics including a mandate to publish ART success rates (Fertility Clinic Success Rate and Certification Act of 1992, Rep. Wyden, Ron 1992). This pressure to have successful births through IVF led the fertility clinics to overproduce embryos and select 'fit' candidates for IVF to increase their success rates. As a result, in 2002 over 400,000 embryos were being cryopreserved by fertility clinics nationwide, with no laws or regulations directing the usage, storage, and disposition of these embryos (Kass 2004).

Research on these embryos became of major interest for fertility clinics and universities. The ability to preserve embryos in laboratory conditions indefinitely allowed scientists to study a variety of developmental biology questions during the earliest stages of human development. The understanding of the neural network and inter-organ connections seemed like a possible emerging field. Other areas of research involving human embryos, such as cloning and creation of human-animal chimeras, remain controversial.

In the latter part of the 20th century, there was a lot of discussion of whether it is permissible to conduct research on embryos; that debate still continues today. Since 1979, the laws, regulations, and guidelines pertaining to human embryos in research have undergone numerous changes. In 1979, the U.S. Department of Health and Human Services (DHHS) disbanded the committee that oversaw research involving human ova, sperm, and embryos. Many of these laws were a direct result of political actions taken as part of the anti-abortion rules (Jones and B.C. 2000). In 1994, a panel of the U.S.'s National Institute of Health (NIH) tried to draft guidelines that directed embryo usage in research, but the guidelines never came to fruition[41].

Meanwhile, in the UK, with the establishment of the "Warnock Committee" in 1982, there was a subsequent inclusion of the 14-day rule into the Human Fertilisation and Embryology Act of 1990 (HFEA). Since then, the HFEA (amended) has been upheld in the country.

[41] "**1994** – The National Institutes of Health convenes a Human Embryo Research Panel to draft up guidelines on ethical funding for research on human embryos. President Bill Clinton overrules the panel's recommendation that funding be permitted for creating embryos for research, but agrees that funding can be used to study embryos leftover from in-vitro fertilization (IVF) procedures"- (Boston's Children Hospital)

Researchers have to procure a license from Human Fertilisation and Embryology Authority to conduct research on human embryos. Human Fertilisation and Embryology Authority also regulates the use of embryos in fertility clinics and the storage of gametes and embryos, conducts inspections, and publishes reports[42,43].

For the establishment of HFEA in early 1990, the Chief Medical Officer's Expert group and the House of Lord's select committee, appointed by the government of the United Kingdom, were charged with the responsibility to advise on regulations for the use of embryos in research. The HFEA states the criteria permitting issue of the license to use embryos in research. It prohibits the use of embryos in research after 14 days and prohibits the implantation of human embryos in an animal (which could lead to the formation of chimeras), among several other rules specifying the particularities of embryo procurement. Section 3 of the Act says:

"(3)A licence cannot authorise—

(a)keeping or using an embryo after the appearance of the primitive streak,

(b)placing an embryo in any animal, [F3 or]

(c)keeping or using an embryo in any circumstances in which regulations prohibit its keeping or use,

F4(d)For the purposes of subsection (3)(a) above, the primitive streak is to be taken to have appeared in an embryo not later than the end of the period of 14 days beginning with

[42] See "How we regulate" (Human Fertilisation and Embryology Authority).
[43] (Aurélie Mahalatchimy 2017) - II Research on Human Embryonic Stem Cells, A) current legal position

[F5] the day on which the process of creating the embryo began], not counting any time during which the embryo is stored."(Human Fertilisation and Embryology Act 1990)

This 1990 Act would permit researchers to use embryos for congenital disease research, research on infertility, increasing knowledge about miscarriage, detecting chromosomal abnormalities and the like. [HFEA Act 1990, Schedule 2, paragraph 3(2)]. Some changes to this were made in 2001[44] that broaden the licensure of embryos to include permitting the usage of embryos for serious disease research or studying the development of the embryo and applying the knowledge in developing treatments for serious disease (Great Britain and Department of Health 2001). This amendment was condemned by bioethicists like Jan Decker, who questioned the loose definition of "serious disease" and expressed discontent with this modification as it, according to him, decreased the value placed on embryos. To him an embryo achieved the moral status of a potential human being on the day of its creation (fertilization)(Deckers 2005).

Laws on creation of chimeras using human cells and animal embryos, and laws on cloning, and how these may help us understand the future of SHEEFs is discussed in the next section. These types of discussions, in the context of SHEEFs, become relevant when we see SHEEFs as a human embryonic cell admixture that can be introduced into a pig or a mouse embryo and allowed to develop – this is a regulatory grey area and more of this is explored in the next section.

[44] Human Fertilisation and Embryology (research purposes) Act, 2001 - (Great Britain and Department of Health 2001)

In addition to ISSCR international guidelines on stem cells and the involvement of ESCROs in regulating embryo research, in the U.S. subpart B of the "common rule" - Federal Policy for the Protection of Human Subjects of 1991 (revised and amended since) (45 C.F.R. §46. 201-207) (DHS 1991) confers protection to fetuses as human subjects during biomedical research. However, the Common Rule does not cover in vitro embryo research that is the focal point of this thesis. Moreover, neither the common rule nor the guidelines address SHEEFs. National Academy of Sciences, National Academy of Engineering, and Institute of Medicine, here on referred to as NASEM, recommended guidelines, that were last amended in 2010, on the ethical use of hESCs in research. These guidelines do not refer to SHEEFs directly, but their recommendations align quite appropriately with what I propose in the thesis, regarding mixing of SHEEFs with animal and human tissues. More about them is discussed in the coming sections.

ii. THE RELEVANCE OF EXISTING LAWS AND GUIDELINES TO SHEEFS

SHEEFs created in a lab to merely understand embryonic development till 14-days may be experimented on according to ISSCR guidelines (which are discussed in the next section), but, at this point SHEEFs being created for the purposes of implantation is a far-fetched idea. Efforts in the future may be made to observe the growth of SHEEFs in-vitro beyond the gastrulation stage and maintain SHEEFs in artificial wombs in the laboratory. Some efforts to do the same with animal embryos have been made e.g. Kuwabara and colleagues from Juntendo University, Japan, successfully gestated goat fetuses in an "extrauterine fetal

incubation system" (Kozuma et al. 1999). For another example, Professor Helen C Liu of Cornell University has been working on developing an artificial environment for the growth of embryos ex-vivo, by studying the function of endothelial cells for organ regeneration (Kedem et al. 2013).

Implantation of SHEEFs in artificial growth environment to study embryonic development seems like a possibility in the future, and this would require SHEEFs not only to be liberated from regulations that apply to human embryos but to be allowed to grow *in vitro* for a much longer duration than 14 days. How far this is ethically acceptable is a question to be explored by the committees or institutions charged with the responsibility to develop guidelines on SHEEFs.

To qualify what is said above about the possibility of gestating SHEEFs and maintaining them in artificial environment to grow into fetuses, there is a need to understand that SHEEFs, although made up of ESCs and extraembryonic markers in Warmflash's lab, do not possess the capacity to divide and self-organize into embryo-like structures by themselves[45]. They would require constant observation and would need to be subjected to different lineage progenitor cells that trigger the growth of germ layers into different tissues and organs as was described in chapter 1. Huarte and Suarez enunciate, in their book, the difference between a living and a "viable" entity – in their terms a) entities that possess vegetative functions and b) entities that are ensouled. According to their stipulations for ensoulment, an entity should possess a "functioning brain capable of spontaneous functions

[45] This does not imply that human embryos have the capacity to divide and differentiate in laboratory conditions all by themselves either – just that SHEEFs need micropatterning and embryos do not.

such as breathing and moving." (Suarez 2011) SHEEFs till this point have not been shown to be capable of performing the functions of even a "viable" entity. Shahbazi et al. told a scientific e-magazine[46] that in would be difficult to see the version of SHEEFs they created in their lab (Shahbazi et al. 2016a), that are very similar to the ones created by Warmflash et. al., "develop in-vitro beyond the stages [they] have characterized."

iii. THE POSSIBILITY OF IMPLANTING SHEEFS AND WHAT THAT ENTAILS

Assuming that SHEEFs continue to successfully mimic stages of canonical embryogenesis post 14 days when allowed to develop, there may be some ostensible ethical restrictions to implant them in animals. It must be borne in mind that such a successful recapitulation would not be possible without studying human embryos post 14 days and comparing the two. This chapter is based on the assumption that researchers find an alternative to studying human embryos to further develop SHEEFs or on the assumption that the 14-day rule is extended in the future[47].

Two views that confer extreme moral status to embryos are: "conceptionalist" ("the embryo is a person") and the strong version of the "potentialist" view (because of the potential of an embryo to become a human, it should be regarded as having the same moral status)(de Wert and Mummery 2003). And an extreme opposite of these two views is the

[46] See the article here by (Paul Tadich 2016)
[47] I do not, however, argue for this in this thesis although most of what has been said would not be possible without an extension of the 14-day rule.

viability-based argument that an embryo (and in some cases, even the fetus) ought not to be thought of as a person because it is not yet viable (i.e. capable of surviving on its own without the help of laboratory equipment or a female's uterus[48]), so, does not possess any moral status. And there are commentaries on the far extreme of not conferring upon human embryos any moral status; for instance, John Harris compared the human embryo to a "Sunday roast" one that can be destroyed for one's nourishment (Dyson and Harris 2012). Commentaries by writers such as George Annas, Julian Savulescu, and Michael Sandel also argue that if embryos were given full moral status, each time embryos are discarded or not implanted, it would be seen as a major health emergency given that there is so much loss naturally, via abortions, and during IVF (Annas 1989; Douglas and Savulescu 2009; Sandel 2005). De Wert and Mummery state that between these two extremes there are a lot of intermediates. Another author Angeliki Kerasidou, argues in her PhD thesis that moral status of embryos need not be an all-or-nothing issue (Kerasidou 2009). SHEEFs, though, should not be subjected to any of these views. I argue that regulations should take into consideration questions like:

- Is the purpose of the experiment to create a viable embryo for implantation? Or,
- to create a genetically stable source of embryonic stem cell-lines? Or,
- to use SHEEFs as a replacement for animal models and human embryos?

If it is the purpose of the experimenter to create an implantable embryo through SHEEFs, the entire argument shifts radically. One must consider the implications of such an

[48] In a different text, the federal register, viability is defined as : "(h) Viable, as it pertains to the neonate, means being able, after delivery, to survive (given the benefit of available medical therapy) to the point of independently maintaining heart- beat and respiration." (§ 46.202 1991)

experiment and more likely than not, such an experiment should not be carried out. Many existing laws already prohibit such a use of embryonic cells. For example, the HFEA amendment of 2008 (section 3ZA) states that a woman may not be implanted with any kind of cluster of cells except a "permitted embryo", which they define to be a product of fertilization of a "permitted egg" by a "permitted sperm"[49]. HFEA also states that a "permitted embryo" is one that contains no other cells, but the cells generated on its own.

If SHEEFs are considered more like human tissues (as they are derived from ESCs) than synthetic embryos, their transplantation would be licensed by the Human Tissues Authority under Human Tissues (Quality and Safety for Human Application) Regulations of 2007 (Q&S Regulations) in the UK[50]. A dozen U.S. states[51] and 60 countries in the world[52] prohibit reproductive cloning and implantation of any entities or admixture of cells other than an embryo (created naturally or through IVF), which would also apply to gestating SHEEFs with the intention to create a human being.

---

[49] 3ZA
"(2) No person shall place in a woman—
(a)an embryo other than a permitted embryo (as defined by section 3ZA), or
(b)any gametes other than permitted eggs or permitted sperm (as so defined)."
"*(4) An embryo is a permitted embryo if—*
(a)it has been created by the fertilisation of a permitted egg by permitted sperm,
(b)no nuclear or mitochondrial DNA of any cell of the embryo has been altered, and
(c)no cell has been added to it other than by division of the embryo's own cells."
(HFEA, 2008)

[50] See (Aurélie Mahalatchimy 2017) and (Organ and Tissue Transplantation Team, Department of Health 2007)

[51] See Embryonic and Fetal Research Laws (NCSL 2016).
[52] (J Reynolds 2014)

iv. CHIMERAS

Another possibility in the much nearer future is the implantation of SHEEFs in animals (primates, rodents, pigs etc.) for growing organs for transplantation. This kind of research involving iPSCs is already under way (e.g. Wu and team performed an experiment by introducing human cells into pig embryos which developed for 28 days (Wu et al. 2017)). In this experiment, the obstacle that was noted by the scientist was that a porcine model may not be an ideal model because the length of the pregnancy in a sow is less than 4 months. In light of experiments like these, it is not completely unforeseen that in the future, where experiments involving the implantation of SHEEFs into animals will be performed.

Currently, there is no federal funding in the United States for studies that involve implantation of human cells and tissues into non-human vertebrate animal pre-gastrulation embryos[53]. As we see in the following sections, this kind of research that involves mixing of human and animal cells is advised more caution in guidelines like those given by NASEM. A bill introduced on Sept 22nd 2016 known as the Human-Animal Chimera Prohibition Act of 2016 (Rep. Smith, Christopher H. [R-NJ-4] 2016) aims to amend title 18 of the United States Code to prohibit human-animal chimeras. Types of research that involve the mixing of hESCs with non-human cells to create "brain derived wholly or predominantly from human neural tissues" would fall under the category of human-animal chimeras, according to this bill (§ 1131, *ibid*).

[53] See notice "…informing the research community that it will not fund research in which human pluripotent cells are introduced into non-human vertebrate animal pre-gastrulation stage embryos while the Agency considers a possible policy revision in this area." (National Institutes of Health 2015)

As the devil is in the details, how embryos and SHEEFs are defined and differentiated will be a major determining factor going forward. Pera points out that "[i]t may even be possible to combine human gastruloids with extra-embryonic cells from animals, so that the chimeric embryos thus created would be essentially human...depending on the definition of the embryo used—such research could result, in the legal sense, in 'upgrading' gastruloids to real (human) embryos." (Pera et al. 2015, 919)

The 2016 bill on the prohibition of human-animal chimeras has recently been opened up for debate and NIH requested a public comment on changes to the existing guidelines on chimeras and lifting the moratorium in place[54]. This may change the likelihood that SHEEFs could be implanted into animals. However, I argue that such studies, posing a risk of creation of human animal chimeras, should not be performed until guidelines on SHEEFs have gone into action.

v. PROFESSIONAL ETHICAL GUIDELINES ON HUMAN EMBRYO RESEARCH

For our purposes to draw a parallel between current embryo research regulations and how new ones can be developed for SHEEFs, it is also important to understand the guidelines on embryo research by professional groups such as ISSCR, scrutinized by EMROs and ESCROs.

[54] (National Institutes of Health 2016)

There are guidelines issued by several professional groups that specify the conditions under which research on pre-implantation embryos may be permissible and morally justifiable. One such professional set of guidelines is written by ISSCR[55]. It holds that "scientific research on preimplantation-stage human embryos is ethically permissible when performed under rigorous scientific and ethical oversight, especially in the areas of human development, genetic and chromosomal disorders, human reproduction, and new disease therapies." It talks about the importance of the source of the stem cells, and if they are human embryos, then the source of those embryos is human, a species that owns many rights of personhood. Principles like "patient respect", "transparency", and "quality of research" are always expected to be supremely adhered to.

In the section 2.I.3.3 CATEGORY 3, ISSCR clearly lists some types of research that at this point shouldn't be allowed to proceed.

2.1.3.3 Category 3. Prohibited research activities. Research under this category should not be pursued at this time because of broad international consensus that such experiments lack a compelling scientific rationale, raise substantial ethical concerns, and/or are illegal in many jurisdictions. Such forms of research include the following:

[55] "These guidelines were prepared by the ISSCR Guidelines Updates Task Force, charged with revising and updating ISSCR Guidelines for the Conduct of Human Embryonic Stem Cell Research (ISSCR, 2006) and Guidelines on the clinical Translation of Stem Cells (ISSCR, 2008). The task force, a group of 25 scientists, ethicists, and experts in health care policy from nine countries, was chaired by bioethicist Jonathan Kimmelman. George Daley and Insoo Hyun, chairs of the guidelines task forces of 2006 and 2008, respectively, provided continuity across the three ISSCR guidelines efforts" (ISSCR 2016, 2)

a. In vitro culture of any intact human preimplantation embryo or organized embryo-like cellular structure with human organismal potential, regardless of derivation method, beyond 14 days or formation of the primitive streak, whichever occurs first.

b. Experiments whereby human embryos or organized cellular structures that might manifest human organismal potential are gestated ex utero or in any non-human animal uterus.

c. Research in which human embryos produced by reprogramming of nuclei from somatic cells by nuclear transfer or comparable techniques are implanted into a human or animal uterus. Given current scientific and medical safety concerns, attempts at human reproductive cloning are prohibited.

d. Research in which human embryos that have undergone modification of their nuclear genome are implanted into or gestated in a human or animal uterus. Genome-modified human embryos include human embryos with engineered alterations to their nuclear DNA and/or embryos generated from a human gamete that has had its nuclear DNA modified, when such modifications will be inherited through the germ line.

e. Research in which animal chimeras incorporating human cells with the potential to form human gametes are bred to each other.

(Page 7 of Guidelines of stem cell research and clinical translations, ISSCR, 2016)

These guidelines (ISSCR 2016 2.1.3.2 Category 2, p7) also states that "[r]esearch involving the in-vitro culture of embryos or experimental generation of embryo-like structures that might manifest human organismal potential, to ensure minimal periods of in-vitro culture, as justified by compelling scientific rationale…" should be subjected to EMRO scrutiny.

As is clear from the text, SHEEFs belong to this category, and as stated in the category quoted previously, gestating them *ex utero* or culturing them beyond 14 days *in vitro* is currently prohibited according to the ISSCR guidelines.

NASEM guidelines, in section 1.3 (c), recommend that types of research that involve mixing of hESCs with non-human primate cells or with other non-human animal cells warrant more attention (Final Report of The National Academies' Human Embryonic Stem Cell Research Advisory Committee and 2010 Amendments to The National Academies' Guidelines for Human Embryonic Stem Cell Research 2010). NASEM guidelines recommend the oversight of ESCRO committees, and they even recommend procuring permission from institutions like IUCAC (Institutional Animal Care and Use Committee), and IBC (Institutional Biosafety Committee), if necessary.

On research that warrants more attention, NASEM guidelines say:

"*The following types of research should not be conducted at this time:*

Research involving in vitro culture of any intact human embryo, regardless of derivation method, for longer than 14 days or until formation of the primitive streak begins, whichever occurs first.

Research in which hES cells are introduced into nonhuman primate blastocysts or in which any embryonic stem cells are introduced into human blastocysts.

In addition:

No animal into which hES cells have been introduced such that they could contribute to the germ line should be allowed to breed."

Another set of such professional guidelines is issued by EMROs (ESCRO[56]s in the U.S.)-Embryo Research Oversight (EMRO) research process entails:

- "*Scientific rationale and merit of proposal. Research with human embryos or embryo-derived totipotent or pluripotent cells requires that scientific goals and methods be scrutinized to ensure scientific rigor. Appropriate scientific justification for performing the research using the specified materials is required.*
- *Relevant expertise of investigators. Appropriate expertise and/or training of the investigators to perform the stated experiments must be ascertained in order to ensure the optimal use of research materials. For derivation of new human embryo-derived cell lines or experiments that involve use of human embryos, relevant expertise would include prior experience with embryo culture and stem cell derivation in animal systems and competence in the culture and maintenance of human embryonic stem cells. Investigators performing derivations of embryo-derived cell lines should have a detailed, documented plan for characterization, storage, banking and distribution of new lines.*
- *Ethical permissibility and justification. Research goals must be assessed within an ethical framework to ensure that research proceeds in a transparent and responsible manner. The project proposal should include a discussion of alternative methods and provide a rationale for employing the requested human materials, including justification*

[56] Embryonic Stem Cell Research Oversight (ESCRO) Committees review research involving human embryonic stem cells (hESC) – each medical center in the U.S. has their own ESCRO they answer to, or work with ESCROs from other medical centers e.g. Albert Einstein College of Medicine has their own ESCRO that oversees embryo related research in their medical center. See: (Albert Einstein College of Medicine 2009)

for the numbers of preimplantation embryos to be used, the proposed methodology, and for performing the experiments in a human rather than animal model system." (ISSCR 2016, 6)

Both examples mention that these guidelines pertain to "embryo-like cellular structure with human organismal potential" in the ISSCR guidelines and "embryo-derived totipotent or pluripotent cells" in EMRO research process which can very well apply to SHEEFs, including the mention of mixing human cells with animal cells in the NASEM guidelines. Currently, SHEEFs are created in the laboratory by adhering to the guidelines on hESCs, which are overseen by EMROs and ESCROs, without ever mixing it with their guidelines on embryos. The research conducted so far on micromanipulation of hESCs with extra-embryonic markers to create SHEEFs was seen as an experiment with embryonic stem cells in vitro only but going forward there would be a need to develop their own class where they are subjected to more stringent scrutiny than hESCs but lesser than embryos.

vi. 14-DAY RULE MODIFICATION IN LIGHT OF THE NEW SHEEFs

As stated in the first chapter, the 14-day rule came to be as a compromise between two groups with opposing views over 40 years ago following the Warnock report. This was named after Mary Warnock, the chair of the IVF Inquiry committee in the 1980s. An earlier mention of the significance of the 14-day rule came up in the U.S. Department of Health,

Education and Welfare Report in 1979[57]. This report, after considering various viewpoints, concluded that 14 days after fertilization, enough events in development like appearance of the PS occur to select it as a practical point for regulation, and therefore, it can be regarded as a significant point, after which point, research on human embryos cannot be morally justified.

There have been efforts to modify the 14-day rule by extending it to 28 days. Although the 14-day rule emerged out of practical reasons, (Hyun, Wilkerson, and Johnston 2016) many would agree that since its creation, moral significance has been ascribed to "14-days" in a variety of ways (Nuffield Council on Bioethics 2016). Giulia Cavaliere wrote that the pluralistic nature of the 14-day rule compromise should be appreciated and maintained because different opinions matter and as long as no one is offended the rule is all encompassing (Cavaliere 2017). The modification of the 14-day rule itself is thus not argued for or suggested in this thesis.

However, SHEEFs are biologically different from human embryos and shouldn't be subject to the 14-day rule. SHEEFs are biologically different from hESC-culture models, too. For example, the ones that were made before Warmflash and colleagues, like by Shahbazi and team, or by Itskovitz-Eldor and colleagues. SHEEFs are by the definition given by Aach et al., and in this thesis, refer to the synthetic embryos developed in a spatial confinement through the addition of extra-embryonic markers. These, as stated earlier, require their own category and a set of new regulations. That said, if the 14-day rule is modified in the future

[57] (U.S. Department of Health, Education and Welfare Report in 1979 1979)

(i.e. extended to 28 days or further), it will have an effect on how SHEEFs are regulated. In this thesis, the basis of the argument is to allow SHEEFs to serve as a category of entities that can be experimented on in ways human embryos cannot be. This contends that SHEEFs have to be allowed to fill the black boxes in human embryogenesis, the terra incognita of developmental biology.

Before the 14th day rule was adopted as a limit for research on embryos, several different options were also considered, each with a moral significance in and of itself (e.g. the 5th day -beginning of implantation in the uterus, and the 11th day - end of implantation, and fertilization) (Wilson 2011). In 1978, after the first successful IVF birth, it was shown that it was possible to maintain human embryos outside of the mother's womb, and given that these embryos could be used for research or for implantation, the Ethics Advisory Board (Department of Health, Education, and Welfare) conducted a detailed consultation and published recommendations in 1979, which were later adopted by the Warnock committee in 1982 (Ethics Advisory Board DoH, Education and welfare 1979). The Warnock committee specified reasons why 14 days was chosen as the criterion, which are listed below.

1) the appearance of the primitive streak which indicates the beginning of gastrulation, which is when the growth occurs in posterior and anterior parts of the embryo (marking the formation of a head-to-tail), and which is also a precursor for brain and spinal cord formation.

2) gastrulation itself is a significant stage where the cells move from a singular type of cells to three different types: the three germ layers

3) the process of twinning: during gastrulation is also when the embryo could cleave and form multiple embryos (i.e. twinning) or the process of two different embryos merging into a single one (i.e. tetragametic chimerism) (Department of Health and Social Security 1984)

These criteria are important for SHEEFs, too, in that bioethicists and scientists have argued for similar criteria for SHEEFs regulation. For instance, Aach et al suggest that these characteristics could occur well before 14 days in SHEEFs and therefore, research on SHEEFs should be terminated whenever these characteristics are observed and not on the 15th day[58].

In the light of the new embryoids, a lot of debate has been sparked both in favor of and in objection to modifying the rule to accommodate these new laboratory creations that clearly don't fit the category of a human embryo. This is different from the existing debate to extend the 14-day rule to allow scientists to conduct research on embryos for longer than 14 days. A lot of scientists and philosophers believe efforts will be made in developmental biology to improve the existing synthetic embryo models such as SHEEFs.

[58] "In particular, we have argued that addressing the issues raised by SHEEFs will require research limits that are based as directly as possible on the presence of early forms of embryonic features that signify moral status." – Page 15 (Aach et al. 2017b)

Rossant and Tam argue that, “At this juncture, the pressing question is whether the development of these embryo-like structures mirrors that of the human embryo *in vivo* closely enough that the outcomes are scientifically relevant to early human development. Further study directly on human embryos is needed to establish a paradigm of developmental correlates to guide the evaluation of the findings from these embryo-like entities.”(Rossant and Tam 2018, 1076) Whether it is possible to rely on the understanding of human embryos to recapitulate embryogenesis in SHEEFs or not, there are many advantages to studying SHEEFs beyond gastrulation – for instance, to create alternatives to embryo destructive research and animal-models, to create a genetically stable source of ESCs, or to use in regenerative medicine. SHEEFs should be studied *in vitro* to learn more about their capacity to be a promising alternative to human embryos. However, their implantation in animals should not be pursued until international guidelines on research on SHEEFs are developed to comply with the existing guidelines on cloning and chimeras.

CONCLUSION

In this thesis, I started with an explanation of emerging hESC-culture models and their various uses in biotechnology such as alternatives to embryo-destructive research, as genetically stable sources of cell-lines, alternatives to animal research, as a way to understand human embryonic development via micropatterning, and in regenerative medicine to list a few. The specific form of hESC-culture model discussed in this thesis is the micropatterned hESC colonies cultured by Warmflash et al. These cell-culture models, known as SHEEFs, were able to mimic human embryo development up to 14 days *in vitro* better than some other models created before. With geometrical confinement of the growth of the cells, these hESC colonies were also able to self-organize, and a PS, which is usually observed in human embryos at 14 days, was also observed. The biological makeup of human embryos, and canonical embryogenesis were noted to understand how SHEEFs were similar to human embryos in some ways and different in others. It was also hypothesized how manipulation of the experimental design in the future could generate SHEEFs that may cause the appearance of a PS earlier than 14 days. Given the differences between SHEEFs and embryos, **I propose a need for a separate category for SHEEFs under hESCs and therefore, also, the need to exclude it from the 14-day rule**.

As of current research, it has not been demonstrated whether it would be possible to grow SHEEFs beyond 14 days *in vitro* as most of the knowledge of micropatterning and administering signals to SHEEFs comes from observing studies performed on mice or on human embryos till 14 days (beyond which it is not possible to study them due to the 14-day rule). And currently, some ways of culturing SHEEFs would be pre-empted by some

guidelines given by professional organizations like ISSCR, where gestating embryo like cell culture models of organismal potential into animals or humans is prohibited. **I propose that culturing SHEEFs beyond 14 days should be encouraged due to the various scientific benefits and due to the safe alternative to embryo-destructive research it provides.** Co-culturing SHEEFs with human extra-embryonic cells should be ventured and after studying them, SHEEFs should be destroyed or stored to be used as a source of stem-cell lines. But, at the same time, **I recommend that, research that aims to implant SHEEFs in artificial wombs, or humans or animals towards a live birth or to harvest organs should not be carried out** as these forms of studies require serious deliberation. Guidelines on SHEEFs should take into consideration the reasons for why human-animal chimeras and reproductive cloning are prohibited and recognize the overlap between some kinds of research on SHEEFs in future and these pre-existing bans.

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