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Modelling human embryogenesis: embryo-like structures spark ethical and policy debate

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BACKGROUND: Studying the human peri-implantation period remains hindered by the limited accessibility of the *in vivo* environment and scarcity of research material. As such, continuing efforts have been directed towards developing embryo-like structures (ELS) from pluripotent stem cells (PSCs) that recapitulate aspects of embryogenesis *in vitro*. While the creation of such models offers immense potential for studying fundamental processes in both pre- and early post-implantation development, it also proves ethically contentious due to wide-ranging views on the moral and legal reverence due to human embryos. Lack of clarity on how to qualify and regulate research with ELS thus presents a challenge in that it may either limit this new field of research without valid grounds or allow it to develop without policies that reflect justified ethical concerns.

OBJECTIVE AND RATIONALE: The aim of this article is to provide a comprehensive overview of the existing scientific approaches to generate ELS from mouse and human PSCs, as well as discuss future strategies towards innovation in the context of human development. Concurrently, we aim to set the agenda for the ethical and policy issues surrounding research on human ELS.

SEARCH METHODS: The PubMed database was used to search peer-reviewed articles and reviews using the following terms: 'stem cells', 'pluripotency', 'implantation', 'preimplantation', 'post-implantation', 'blastocyst', 'embryoid bodies', 'synthetic embryos', 'embryo models', 'self-assembly', 'human embryo-like structures', 'artificial embryos' in combination with other keywords related to the subject area. The PubMed and Web of Science databases were also used to systematically search publications on the ethics of ELS and human embryo research by using the aforementioned keywords in combination with 'ethics', 'law', 'regulation' and equivalent terms. All relevant publications until December 2019 were critically evaluated and discussed.

OUTCOMES: *In vitro* systems provide a promising way forward for uncovering early human development. Current platforms utilize PSCs in both two- and three-dimensional settings to mimic various early developmental stages, including epiblast, trophoblast and amniotic cavity formation, in addition to axis development and gastrulation. Nevertheless, much hinges on the term 'embryo-like'. Extension of traditional embryo frameworks to research with ELS reveals that (i) current embryo definitions require reconsideration, (ii) cellular convertibility challenges the attribution of moral standing on the basis of 'active potentiality' and (iii) meaningful application of embryo protective directives will require rethinking of the 14-day culture limit and moral weight attributed to (non-)viability. Many conceptual and normative (dis)similarities between ELS and embryos thus remain to be thoroughly elucidated.

WIDER IMPLICATIONS: Modelling embryogenesis holds vast potential for both human developmental biology and understanding various etiologies associated with infertility. To date, ELS have been shown to recapitulate several aspects of peri-implantation development, but critically, cannot develop into a fetus. Yet, concurrent to scientific innovation, considering the extent to which the use of ELS may raise moral concerns typical of human embryo research remains paramount. This will be crucial for harnessing the potential of ELS as a valuable research tool, whilst remaining within a robust moral and legal framework of professionally acceptable practices.

Key words: embryogenesis / stem cells / embryonic stem cells / trophoblast stem cells / blastoids / gastruloids / ethics / embryo-like structures / embryoids / pluripotency

Introduction

Hindered by the inaccessibility of the *in vivo* environment, scarcity of research material and inherent ethical and legal constraints, studying the peri-implantation period in human remains a daunting task. To elucidate the complexities of embryonic development, continued efforts have been directed towards generating models that recapitulate embryogenesis *in vitro*. Notably, pluripotent stem cell (PSC)-based embryo-like models have taken precedence in complementing *in vivo* studies, contributing to the newly emerging field of synthetic developmental biology (Ebrahimkhani and Ebisuya, 2019).

In contrast to *in vivo* or *in vitro* embryos resulting from the process of fertilization, the entities at issue here are formed through stem cell coaxing, providing an amendable tool for mimicking developmental processes. This also implies a second difference in comparison to fertilization-based embryos, namely that their genome is not individually unique, but rather represents a genetic clone of the stem cells and/or donor somatic cells of origin. The general term for these entities is still under discussion, varying between embryoids, synthetic or artificial embryos and synthetic entities with human embryo-like features (SHEEFs). Since some of these terms have either already been used in different contexts or prematurely denote these entities as embryos, we herein propose referring to stem cell-based embryo models as 'embryo-like structures' (ELS) to avoid misconceptions.

The considerable plasticity for modelling embryogenesis is not only alluring for human developmental biology but also holds vast potential for improving clinical approaches in assisted reproductive technologies (ART). Nevertheless, the features that make these structures scientifically interesting, also give rise to ethical and regulatory issues. It thus remains to be established whether ELS in fact represent a morally preferable alternative to research with human embryos.

The overall aim of the present article is two-fold. We endeavour to (i) provide an overview of the scientific approaches to generate ELS in both mouse and human, while discussing future strategies towards innovation in the context of human development and (ii) set the agenda for the ethical and policy issues raised by the generation, culture and use of ELS in a research context. We do so by drawing on prominent conceptual and normative insights from the human embryo research debate and inferring what they may imply for (research with) ELS.

Embryonic development in mice and humans

Mammalian development begins with the formation of the zygote (Fig. 1). During the first days of development, the zygote undergoes regular mitotic divisions, yet transcriptional activity relies almost exclusively on maternal messenger RNA. The embryo undergoes a major wave of embryonic genome activation at the two-cell stage in mouse and at the 4- to 8-cell stage in human (Vassena *et al.*, 2011; Niakan and Eggan, 2013), an essential process for directing further developmental programmes.

Compaction begins at the morula stage, at the late 8-cell stage (embryonic day (E)2.5) in mouse and 16-cell stage (day 4) in human (Table I, Fig. 1). The compacted morula then starts to absorb fluid, establishing the blastocoel cavity as the hydrostatic pressure increases. This culminates in the formation of the blastocyst. At this stage, the inner placed blastomeres form the inner cell mass (ICM) at one side of the cavity, while those at the periphery establish the trophectoderm (TE), a thin single-layered epithelium. At E4.5 in mouse, the ICM further segregates into pluripotent epiblast progenitor cells (eventually forming the embryo proper) and the hypoblast or primitive endoderm (PE) which predominately contributes to the yolk sac endoderm (Fig. 1) (Wamaitha and Niakan, 2018). While these lineage contributions are likely to also apply in the human context (Yan *et al.*, 2013; Petropoulos *et al.*, 2016), this process is yet to be elucidated. Interestingly, *in vitro* culture itself may influence lineage segregation dynamics in human. Blastocyst morphology, including ICM and TE quality, was shown to be significantly reduced in *in vitro* cultured embryos, compared to *in vivo* counterparts (Munné *et al.*, 2020).

In both mouse and human, the epiblast, PE and TE lineages are each marked by the expression of specific genes (Fig. 1). While some lineage-specific markers are conserved between mouse and human, many others show species-specific expression dynamics (Fig. 1) (Roode *et al.*, 2012; Niakan and Eggan, 2013; Blakeley *et al.*, 2015; Deglincerti *et al.*, 2016b). For instance, while TFAP2C is enriched in the mouse TE, it is expressed at similar levels in both the TE and epiblast in human. *CLDN10* and *PLAC8* are highly expressed in the human TE and not expressed in mouse TE, while *Eomes* is enriched in the mouse TE and completely absent in human (Blakeley *et al.*, 2015). Additionally, *Cdx2* expression is specific to TE cells in mouse, yet shows variable expression in human (Niakan and Eggan, 2013). Moreover, *FOXA2* is specific for human PE, while it is not expressed in mouse blastocysts (Fig. 1). These differences highlight the diverging regulatory mechanisms underlying human preimplantation development compared to mouse.

Preimplantation development takes about 5–6 days in human and 4.5 days in mice. At this time, the blastocyst begins to hatch out of the zona pellucida and is ultimately able to interact directly with the maternal endometrium *in vivo*. Mammalian implantation relies on the developmental synchrony between the decidualized endometrium and the TE, while the peri-implantation microenvironment further mediates blastocyst attachment and invasion (Table I). These processes are regulated by factors secreted from both the maternal uterus and the embryo (Cha *et al.*, 2012; Gellersen and Brosens, 2014; Salamonsen *et al.*, 2016). This intricate discourse is crucial for embryonic development and ultimately the successful initiation of pregnancy.

Following implantation, developmental progression appears to be less conserved between mouse and human (Fig. 1). The mouse embryo elongates to form the characteristic egg-cylinder, with a cup-shaped epiblast, while in human, the epiblast remains flat or disc shaped (Fig. 1). In mice, the polar TE is reshaped to form the extraembryonic ectoderm (which is absent in human) and the ectoplacental cone, which eventually contributes to the chorion and the placenta. In mice, both the extraembryonic ectoderm and the epiblast form cavities, which fuse into the pro-amniotic cavity (Fig. 1). Specific to rodents, parietal endoderm differentiates and migrates from the PE and lines the TE, separated by a basement membrane. The structure is known as Reichert's membrane and functions as the parietal yolk

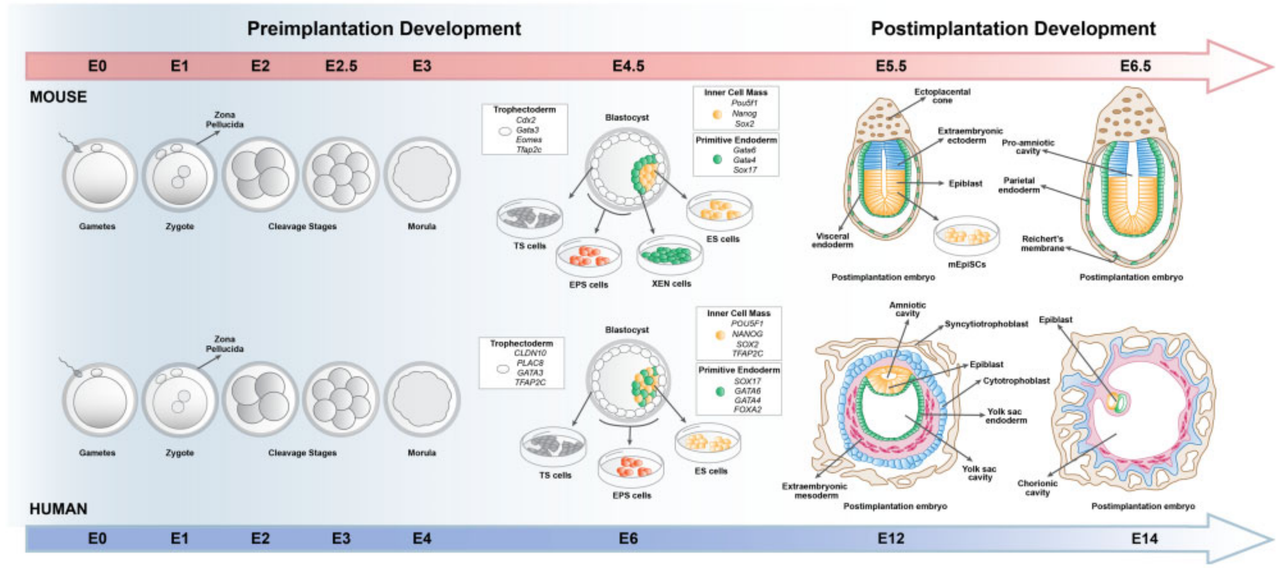


Figure 1. Overview of peri-implantation development in mouse and human. Lineage-specific markers of the trophectoderm, inner cell mass and primitive endoderm in both mouse and human are shown. EPS cells, extended pluripotent stem cells; ES cells, embryonic stem cells; mEpiSCs, mouse epiblast stem cells; TS cells, trophoblast stem cells; XEN cells, extraembryonic endoderm cells.

Table 1 Table of definitions.

Scientific terminology	Definition
Embryo-like structure(s)	Umbrella term for pluripotent stem cell-based embryo models that resemble the morphology and physiology of fertilization-based embryos, as proposed by the authors.
Compaction	During compaction the originally round and loosely adherent cells of the embryo flatten, developing a polarity that maximizes contact between blastomeres. This reorganization involves the activity of cytoskeletal and cell adhesion elements.
Axis formation	The three germ layers (endoderm, mesoderm and ectoderm) are organized relative to a coordinate system (anterior–posterior, dorsal–ventral and left–right axes). This acts as a reference for the development and patterning of tissues and organs.
Gastrulation	In amniotes, gastrulation is the process by which the three definitive germ layers ectoderm, endoderm and mesoderm are formed from the epiblast. Each germ layer is lineage restricted and gives rise to specific organs.
Primitive streak (PS)	The appearance of the PS marks the initiation of gastrulation. The PS is a transient structure that marks the posterior or caudal part of the longitudinal axis and bilateral symmetry in amniotes. The PS functions to channel epiblast cells fated to become (embryonic) mesoderm and (definitive) endoderm, which ingress to establish the germ layers. Cells of the epiblast at (or ingressing through) the primitive streak undergo an epithelial-to-mesenchymal transition.
Cardiogenesis	Cardiogenesis (or heart development) begins with the formation of the heart tube and the initiation of a heartbeat around 22 days post-fertilization in humans and embryonic day 8.0–8.5 in mice.
Neurulation	Neurulation denotes the transition from the neural plate to the neural tube, the embryonic precursor of the brain and spinal cord. The closure of the neural tube (with closure of both cranial and caudal neuropore) is completed 26 days post-fertilization in humans and at embryonic day 10.5 in mice.
Somitogenesis	Somitogenesis is the process by which two longitudinal rows of (paraxial) mesoderm flanking the neural groove condense into two strings of blocks called somites. The somites develop into (parts of) the skeleton, musculature and dermis. Somitogenesis takes place from around 21 days post-fertilization in humans and embryonic day 8.0 in mice. Humans develop about 42–44 pairs of somites and this process is completed around 30 days post-fertilization.
Twinning	Twinning generally stems from two situations during development. Dizygotic twins arise from the fertilization of two oocytes, which implant separately. In contrast, monozygotic twins are the results of a single fertilized oocyte splitting during early embryonic development. In monozygotic twinning: (i) if the splitting occurs during the cleavage stage, monozygotic twins will develop separate foetal membranes and placentas; (ii) if the inner cell mass splits, twins will be surrounded by separate amnions, but share the same chorion/placenta; and (iii) if the bilaminar disc splits, the twins will occupy the same amnion and share the same chorion/placenta. This last type of twinning can occur up to 14–15 days post-fertilization.
Totipotency	Totipotency refers to the ability of a single cell to divide and generate all specialized cells of an organism, including embryonic and extraembryonic tissues. Accordingly, the zygote and individual cells of the cleavage stage embryo are the ultimate totipotent cells, as they can give rise to an entire organism.
Pluripotency	Pluripotency refers to the potential of a cell to generate embryonic or adult cell types, both *in vitro* and *in vivo*. The cells of the inner cell mass are pluripotent, as they maintain the potential to differentiate into any of the three germ layers: endoderm, mesoderm or ectoderm. Pluripotency can be captured *in vitro* through the derivation of embryonic stem cells or by direct reprogramming of somatic cells.
Multipotency	Multipotency refers to the potential of a cell to differentiate into a limited number of cell fates.
Somatic cell nuclear transfer (SCNT)	The developmental potential of somatic cells can be restored to totipotency by SCNT. SCNT is the process of transferring nuclear DNA from a donor somatic cell into a recipient enucleated mature oocyte, which can further give rise to an embryo. The SCNT-derived embryo can be used for the derivation of embryonic stem cells, which are genetically identical to the original somatic cell. This process is also referred to as therapeutic cloning.
Induced pluripotent stem cells (iPSCs)	iPSCs are generated by reprogramming somatic cells into a pluripotent state similar to that of embryonic stem cells, by the forced overexpression of four transcription factors: Pou5f1, pou class 5 homeobox 1; Sox2, SRY-Box 2; Klf4, kruppel like factor 4; and c-Myc, c-myc. Induced PSCs overcome the need to use embryos for pluripotent stem cell derivation. Like SCNT-embryonic stem cells, iPSCs are genetically identical to the original somatic cell from which they are derived.
Embryonic stem cells (ESCs)	ESCs are pluripotent stem cells generally derived from the blastocyst inner cell mass.
Naive and primed pluripotency	Embryonic stem cells are known to exist in at least two states of pluripotency: naive and primed. Although derived from the same blastocyst stage inner cell mass, human and mouse embryonic stem cells show different properties reflecting these two states. Accordingly, mouse embryonic stem cells adopt the naive state, more similar to the preimplantation epiblast, while human embryonic stem cells are in a primed state, more akin to the post-implantation epiblast. Pluripotent stem cells in these two states show differing morphologies, distinct culture requirements

(continued)

Table I Continued

Scientific terminology	Definition
	and different transcriptional and epigenetic signatures. Swapping culture conditions can convert naive pluripotent stem cells to the primed state and vice-versa.
Extraembryonic endoderm (XEN) cells	XEN cells are multipotent stem cells that recapitulate properties of the extraembryonic visceral endoderm. To date, XEN cells have not been successfully derived from human embryos, however naive human embryonic stem cells have been shown to acquire characteristics of the extraembryonic endoderm in response to Wnt, Nodal and LIF signalling.
Trophoblast stem cells (TSCs)	TSCs are multipotent stem cells that recapitulate properties of the extraembryonic trophectoderm. In mouse, TSCs can be derived directly from blastocysts but can also been induced (iTSCs) by reprogramming somatic cells using forced overexpression of four transcription factors: Tfap2c, transcription factor AP-2 gamma; Gata3, GATA-binding protein 3; Eomes, eomesodermin; and Ets2, E26 avian leukaemia oncogene 2,3′ domain. In human, TSCs can be derived from blastocysts and first trimester placentas.
Extended pluripotent stem cells (EPS)	EPS are cells that exhibit totipotent-like developmental potential. They should harbour the capacity to contribute to both embryonic and extraembryonic lineages.
Embryoid bodies (EBs)	EBs are three-dimensional aggregates differentiated from pluripotent stem cells with the purpose of obtaining cells of the three germ lineages *in vitro*.
Epithelial-to-mesenchymal transition (EMT)	EMT involves the transition of polarized epithelial cells towards motile apolar mesenchymal cells.
Decidualization	Decidualization of the human endometrium involves extensive morphological and functional differentiation of the endometrial stromal cells that begins approximately 6 days after ovulation. This process is critical as impairment results in implantation failure, recurrent miscarriage or pregnancy disorders, ultimately leading to infertility. Decidualized endometrial stromal cells provide nutrition to the implanting blastocyst and support further peri-implantation development.
Implantation	Implantation involves the attachment and invasion of the embryo within the maternal endometrium. The initial attachment phase encompasses apposition of the endometrial epithelia and trophectoderm of the embryo, followed by adhesion amongst these epithelial surfaces. Subsequent invasion of the trophectoderm occurs through the endometrial luminal epithelium, which allows the embryo to embed within the endometrial stroma. Factors secreted at the embryo-maternal interface are critical for supporting the implantation process and for establishing a successful pregnancy.

Legal terminology	Definition
Human embryo (Research Involving Human Embryos Act, Australia (2002))	'. . . a discrete entity that has arisen from either:(a) the first mitotic division when fertilization of a human oocyte by a human sperm is complete; or (b) any other process that initiates organized development of a biological entity with a human nuclear genome or altered human nuclear genome that has the potential to develop up to, or beyond, the stage at which the primitive streak appears; and has not yet reached 8 weeks of development since the first mitotic division'.
Human embryo (Embryos Act, The Netherlands (2002); Medically Assisted Reproduction and the Disposition of Supernumerary Embryos and Gametes Act, Belgium (2003))	". . . a cell or cluster of cells with the potential to develop into a human being."
Human embryo (Law 14/2007 on Biomedical Research, Spain (2007))	'. . . a phase of embryonic development from the moment in which the fertilized oocyte is found in the uterus of a woman until the beginning of organogenesis, and which ends 56 days from the moment of fertilization, with the exception of the computation of those days in which the development could have been stopped'.
Human embryo (Human Fertilisation and Embryology Act, United Kingdom (1990))	'(a) . . . a live human embryo where fertilization is complete, and (b) references to an embryo include an egg in the process of fertilization, and, for this purpose, fertilization is not complete until the appearance of a two-cell zygote'.
Human embryo (Human Fertilisation and Embryology Act, United Kingdom (2009))	'(a) . . . a live human embryo and does not include a human admixed embryo, and; (b) references to an embryo include an egg that is in the process of fertilization or is undergoing any other process capable of resulting in an embryo'.
Human embryo (Embryo Protection Act, Germany (2011))	'. . . the human oocyte, fertilized and capable of developing, from the time of the fusion of the nuclei, and further, each totipotent cell removed from an embryo that is able to divide and to develop into an individual under the appropriate conditions for that'.
Pre-embryo (Law 14/2007 on Biomedical Research, Spain (2007))	'. . . the embryo constituted *in vitro*, derived from the group of cells resulting from the progressive division of the oocyte until 14 days after fertilization'.

Ethical terminology	Definition
Conceptual analysis	The activity of clarifying what concepts mean and imply.
Normative analysis	The activity of producing or assessing the soundness and justifiability of arguments with reference to normative (moral or legal) questions.

(continued)

Table I Continued

Ethical terminology	Definition
Regulatory analysis	The activity of examining rules or systems (e.g. legal norms, professional codes of conduct, etc.) relevant to an activity or process.
The proportionality criterion	The ethical principle according to which the burdens of an activity (e.g. research) must be proportional to the benefits it yields for it to be morally acceptable.
The subsidiarity criterion	The ethical principle according to which the benefits of an activity (e.g. research) must be acquired through the morally least problematic means for it to be morally acceptable.
Active (inherent) potentiality	The view that the potential to develop into a human being is the *inherent power* possessed by the human embryo to undergo changes to itself.
Passive (contingent) potentiality	The view that the potential to develop into a human being is a *contingent possibility* that depends upon a series of external events and/or actors.

sac, providing nutrients to the developing embryo (Fig. 1). At this point, symmetry breaking starts and the distal tip of the visceral endoderm migrates to the prospective anterior part of the embryo, acting as a signalling centre to define the anteroposterior axis (Takaoka *et al.*, 2011). In humans, the amniotic cavity is immediately formed as the epiblast epithelializes and cavitates, early during implantation (Fig. 1). Moreover, the extraembryonic mesoderm forms by day 12 post-fertilization in human, contributing to the chorionic cavity (Dobreva *et al.*, 2010) (Fig. 1). In contrast to humans, in mice, the amnion, yolk sac and chorion are only formed following the initiation of gastrulation (De Sousa Lopes and Mummery, 2014). In both humans and mice, the germline is set aside just before gastrulation, with the formation of the primordial germ cells (Tang *et al.*, 2016; Popovic *et al.*, 2019).

Gastrulation serves as the gateway to shaping the body plan. During this process, the primitive streak (PS) coordinates extensive cell rearrangements that culminate in the formation of the three germ layers (endoderm, mesoderm and ectoderm) (Table I). Gastrulation is shortly followed by cardiogenesis, neurulation and initiation of somitogenesis (Table I).

Fertilization-based embryos: a foundation for understanding early human development

Hertig and Adams (1967) and Hertig and Rock (1973) were the first to observe and characterize early human embryos *in vivo*. Since then, our fundamental understanding of early human development has primarily stemmed from in vitro fertilization (IVF) in the context of ART. Although limited to the preimplantation period, IVF has certainly provided valuable morphokinetic and metabolic insights into human embryogenesis. However, the signalling interactions and fate diversification mechanisms that accompany these events remain obscure.

To investigate human post-implantation development, efforts have enabled the extended *in vitro* culture of human embryos beyond the implantation stages. O'Leary and colleagues were the first to examine whole human embryos cultured beyond the blastocyst stage *in vitro* during stem cell derivation, up to 13 days post-fertilization. The reported outgrowths contained the post-ICM intermediate, a structure presumed to closely resemble the human peri-implantation epiblast (O'Leary *et al.*, 2012; O'Leary *et al.*, 2013). Based on mouse systems of post-implantation development (Hsu, 1979; Bedzhov *et al.*, 2014), subsequent models allowed extended culture up to 14 days, further attesting to the remarkable ability of the early embryo to self-organize, even in the absence of maternal tissues (Deglincerti *et al.*, 2016b; Shahbazi *et al.*, 2016). Embryo outgrowths showed comparable morphology to early *in vivo* post-implantation human embryos. Specifically, a clear distinction between the epiblast and PE was observed, while putative amniotic and yolk sac cavities were also described (Deglincerti *et al.*, 2016b; Shahbazi *et al.*, 2016). Nevertheless, due to the flattened structure of the outgrowths, these cavities did not expand.

The ability to culture embryos beyond the implantation stages sparked debate regarding the 14-day culture rule for research on human embryos *in vitro* (Hyun *et al.*, 2016). This time point signifies the formation of the PS in human embryos and is also the last stage at which twinning may occur, or at which two embryos can merge (i.e. tetragametic chimerism) (Table I). Nevertheless, the possibility of extended *in vitro* human embryo culture beyond the PS stage remains unknown. Although extended culture systems are a powerful tool for studying the peri-implantation period, certain limitations still persist (Rossant, 2016). The central drawback of the established models is that embryo outgrowths are predominately flattened, which considerably confounds identification of 3D structures formed during normal embryogenesis. Further improvements in culture conditions and the use of refined extracellular matrices may allow greater precision in mimicking physiological conditions. Models involving other species, evolutionarily closer to human, are more amenable to high-resolution technologies, recently providing interesting new data (du Puy *et al.*, 2011; Kuijk *et al.*, 2012; Sasaki *et al.*, 2016; Kobayashi *et al.*, 2017; Ma *et al.*, 2019; Niu *et al.*, 2019; Taniguchi *et al.*, 2019).

In vitro models of embryogenesis

The study of embryogenesis is traditionally based on observing and manipulating human and animal embryos directly. In contrast, the field of synthetic embryology is focused on building ELS from different PSC types *in vitro*. Modulating pluripotency pathways has supported the generation of PSC lines of specific embryonic origin (Fig. 1, Table I).

These have been utilized in two-dimensional (2D) and three-dimensional (3D) settings to mimic various stages of early development (Table II).

Capturing cellular potency *in vitro*

Progress in PSC research started with the observation that teratocarcinoma cells were able to form heterogeneous cell masses (tumours) containing differentiated tissue-like structures following intraperitoneal transplantation in mice (Pierce and Dixon, 1959; Stevens, 1959). Accordingly, mouse embryonal carcinoma cells were the first PSCs described (Kleinsmith and Pierce, 1964; Martin and Evans, 1975). These discoveries inspired the derivation of both mouse (mESCs) and human embryonic stem cells (hESCs) from blastocysts (Evans and Kaufman, 1981; Martin, 1981; Thomson *et al.*, 1998) (Fig. 1). Remarkably, however, hESCs required different culture conditions and showed a distinct epithelial morphology, compared to mESCs. Unlike mESCs that displayed characteristics similar to the preimplantation ICM, hESCs expressed genes of the post-implantation epiblast. Several years later, mouse epiblast stem cells (mEpiSCs) derived from post-implantation embryos, were described to share many similarities with hESCs, including culture conditions, epigenetic state and marker genes (Brons *et al.*, 2007; Tesar *et al.*, 2007). Ultimately, this led to the view that pluripotency exists in at least two states, naive (mESCs) and primed (hESCs and mEpiSCs) (Table I). In subsequent years, the paradigm of obligatory directional differentiation changed with the discovery of human somatic cell nuclear transfer (SCNT) (Tachibana *et al.*, 2013) and particularly, the reprogramming of differentiated somatic cells into induced pluripotent stem cells (iPSCs) (Takahashi *et al.*, 2007) (Table I). With this technology, many new opportunities for developing patient-specific applications were explored.

The establishment of PSCs (ESCs and iPSCs) made it possible to further characterize pluripotency and study the molecular mechanisms of differentiation *in vitro*. PSCs have the potential to contribute towards all embryonic derivatives; however, they are limited in their capacity to form extraembryonic tissues. In mouse, the developmental potency of the PE and TE have been captured following the derivation of extraembryonic endoderm stem cells (XEN cells) (Kunath *et al.*, 2005) and trophoblast stem cells (TSCs) (Tanaka *et al.*, 1998) (Fig. 1, Table I). In human, self-renewing TSCs were established from both human blastocysts and villous cytotrophoblast cells (Okae *et al.*, 2018) (Fig. 1). The resulting human TSCs resemble primary trophoblast cells, however further functional analysis will reveal their likeness to *in vivo* counterparts. Interestingly, primary human TE-like spheroids have been generated from hESCs and provide a useful model for evaluating embryo attachment to various endometrial-like cells (Lee *et al.*, 2015). Nevertheless, the extent to which the adhesion of TE-like spheroids truly mimic the complex mechanisms employed by the blastocyst remains to be elucidated (Weimar *et al.*, 2013). Additionally, a recent report described the derivation of extended PSC (EPS) lines in both mouse and human (Yang *et al.*, 2017). EPS were shown to have a totipotent-like developmental potential, maintaining the capacity to contribute towards both embryonic and extraembryonic lineages (Yang *et al.*, 2017) (Table I). Remarkably, expandable PE-like cells have also been obtained in human, albeit only from hESCs, showing characteristics of extraembryonic endoderm, similar to mouse XEN cells (Table I). However, as these cells were derived from hESCs *in vitro*, their exclusive extraembryonic character remains difficult to verify functionally (Linneberg-Agerholm *et al.*, 2019).

Modelling mouse preimplantation development: blastocyst-like structures

The establishment of different PSCs *in vitro* has been fundamental for modelling preimplantation development. Nevertheless, current models remain limited to the mouse. In particular, cultured PSCs have been

Table II **2D and 3D settings to mimic early embryonic stages.**

Developmental stage	Embryo-like structure	Composition	Species	Study
Preimplantation	ETS-blastoids	PSCs and TSCs	Mouse	Rivron *et al.* (2018)
	Induced blastocyst-like cysts (iBLCs)	PSCs	Mouse	Kime *et al.* (2019)
	EPS-blastoids	EPS cells	Mouse	Li *et al.* (2019)
Post-implantation/gastrulation	ETS-embryos	PSCs and TSCs	Mouse	Harrison *et al.* (2017)
	ETX-embryos	PSCs, TSCs, XEN cells	Mouse	Sozen *et al.* (2018) Zhang *et al.* (2019)
	Gastruloids (micropatterned PSC colonies)	PSCs	Human	Warmflash *et al.* (2014) Etoc *et al.*, (2016) Yoney *et al.* (2018) Martyn *et al.* (2018) Tewary *et al.* (2019)
	Post-implantation amniotic sacembryoid (PASE)	PSCs	Human	Shao *et al.* (2017) Zheng *et al.* (2019)
	Gastruloids (3D elongated EBs)	PSCs	Mouse	van den Brink *et al.* (2014) Beccari *et al.* (2018) van den Brink *et al.* (2020)

PSC(s), pluripotent stem cell(s); TSCs, trophoblast stem cells.

used to generate blastocyst-like structures (also referred to as blastoids or induced blastocyst-like cysts, iBLCs) (Rivron *et al.*, 2018; Kime *et al.*, 2019; Li *et al.*, 2019) (Table II).

Interestingly, blastocyst-like structures were first observed, following the derivation of mouse germ cells *in vitro* (Hübner *et al.*, 2003). Mouse ESCs developed into mature oocyte-like cells that later formed *in vitro* structures resembling mouse preimplantation embryos. More recently, Rivron *et al.* (2018) reported the generation of mouse blastoids by the sequential aggregation of mESCs and mouse TSCs (mTSCs), later referred to as ETS-blastoids (Li *et al.*, 2019). ETS-blastoids were generated in microwell arrays and resemble mouse blastocysts in terms of size, morphology and gene expression. Upon introduction into the uterus of pseudo-pregnant mice, ETS-blastoids induced decidualization, however poorly developed into post-implantation structures both *in vivo* and *in vitro*. As such, these structures are not viable and cannot be used for studying post-implantation development. Their incapacity to develop beyond the implantation stages may be attributed to a defect in the PE, which, as the authors demonstrated, did not develop properly (Rivron *et al.*, 2018). A recent preliminary study, in which the blastoid culture was optimized to form a PE-like compartment more efficiently, indeed showed enhanced survival and morphogenesis of blastoid-derived post-implantation ELS *in vitro* (Vrij *et al.*, 2019). Possibly a critical factor resulting from the absent PE is the related absence of the Reichert's membrane and parietal yolk sac.

Kime *et al.* (2019) further generated blastocyst-like structures starting from mPSCs only. The formation of iBLCs relied on the establishment of progenitor cells that express the totipotency-related, cleavage-stage marker MERVL (Macfarlan *et al.*, 2012). Like ETS-blastoids, the generated iBLCs morphologically resembled mouse blastocysts, induced decidualization in pseudo-pregnant mice and similarly did not develop further (Kime *et al.*, 2019). A further study revealed that it is also possible to generate blastocyst-like structures from mouse EPS (mEPSCs). EPS-blastoids, consist of all three cell lineages (epiblast, TE and PE cells) (Li *et al.*, 2019). Like ETS-blastoids and iBLCs, EPS-blastoids also resembled mouse blastocysts in terms of size and morphology, showed potential to induce decidualization in pseudo-pregnant mice but underwent resorption a few days after transfer. Interestingly, the authors were also able to generate clonal EPS-blastoids from a single EPS cell, albeit at a very low efficiency (2.7%). EPS-blastoids were also generated from iPSCs, serving as a proof of principle that blastocyst-like structures can be generated from somatic cells (Li *et al.*, 2019).

Preimplantation ELS may serve as a valuable model for elucidating the expansion of the mouse blastocoel cavity and the interaction between mPSCs and mTSCs. As aforementioned, blastocyst-like structures have not been developed from human cells thus far; however, research in coming years is likely to follow a similar path as in mouse.

Modelling mouse post-implantation development: ETS/X embryos

Aspects of mouse post-implantation development have also been captured *in vitro*. Aggregates of mESCs and mTSCs cultured in a 3D extracellular matrix-like scaffold have been shown to self-assemble and acquire a cylindrical architecture, similar to that of the early post-implantation embryo (Harrison *et al.*, 2017). These structures were coined ETS-ELS (ESC and TSC ELS). ETS-ELS showed signs of asymmetric induction of mesoderm and specification of cells resembling primordial germ cells in their gene expression patterns. However, due to the absence of extraembryonic endoderm, further (proximal–distal) patterning could not be achieved. In a further study, the combination of mESCs, mTSCs and mouse XEN cells led to ELS that recapitulated gastrulation-like events, including anterior–posterior and proximal–distal patterning, epithelial to mesenchymal transition (EMT) (Table I, Table II) and the formation of definitive endoderm (Sozen *et al.*, 2018). These so-called ETX-ELS (ESC, TSC and XEN cell ELS) aggregated spontaneously under shaking-conditions (Zhang *et al.*, 2019), indicating a certain level of self-organizing properties of mouse development. Following transfer into pseudopregnanct mice, ETS or ETX-ELS-induced decidualization, but degraded after 3 days (Zhang *et al.*, 2019). Ultimately, this is to be expected as mouse implantation starts at the blastocyst stage, resulting from the interaction of mural TE cells with the endometrium. These cells are not maintained in the ETS and ETX-ELS. Moreover, this highlights the importance and robust nature of the decidualization reaction.

To date, the generation of human ETS or ETX-ELS has not been described. Due to the geometrical differences between mouse and human peri-implantation embryos, it would be preferable to develop a model with hESCs and XEN cells inside a layer of TSCs, instead of hESCs and TSCs inside a layer of XEN cells, used to model the mouse.

Modelling human post-implantation development: micropatterns

Manipulating the microenvironment of differentiating PSCs by confining them to micropatterns or within 3D networks has been shown to simulate early morphogenesis *in vitro* in a more reproducible manner. It has been established that cell fate can depend on the size of stem cell colonies (Lee *et al.*, 2009) and research efforts have been directed towards culturing PSCs in geometrically confined colonies (Peerani *et al.*, 2009). For this, cells are deposited on circular-shaped micropatterns to which they can attach (Deglincerti *et al.*, 2016b). Generating micropatterned colonies ensures both reproducibility, as well as a quantitative platform for (imaging) analysis using well-established algorithms to determine lineage restriction and cell movement in real time. Using this method, Warmflash *et al.* (2014) demonstrated that micropatterned colonies of hESCs (also referred to as human gastruloids) cultured in the presence of BMP4 acquired fates of the three germ layers and TE, which were radially organized. Moreover, cell fate depended on the distance from the colony edge, coinciding with the localization of BMP4 receptors (Warmflash *et al.*, 2014; Etoc *et al.*, 2016; Yoney *et al.* 2018). In a further study, micropatterns were used to construct a rudimentary fate map of the human PS. However, in the absence of human data, comparisons with the mouse embryo were used to define markers for human cell types (Martyn *et al.*, 2019).

Overall, micropatterns have proven advantageous for studying size regulation and signalling requirements of hPSCs during gastrulation. However, the morphology of these 2D cultures does not resemble that of the human embryo, in which the three germ layers are positioned on top of each other, with a surrounding TE. Moreover, bilateral symmetry was not established *in vitro*. Nevertheless, these models have certainly set the stage for future work using geometrical control to study differentiation dynamics in a highly quantitative manner. Work on combining PSCs with synthetic or tissue-derived scaffolds in combination with sophisticated bioengineering methods may ensure greater feasibility for modelling the development of complex tissues.

Modelling human post-implantation towards gastrulation: 3D ELS

Research encompassing 3D models of the human embryo has also achieved remarkable progress in recent years. In human, advancements have been made in generating structures mimicking the amniotic cavity. Shao *et al.* (2017) modelled amniogenesis by using a 3D culture system to induce hESCs and hiPSCs to self-organize into structures that closely resembled the human amnion and ectoderm, termed 'post-implantation amniotic sac embryoid' or PASE (Shao *et al.*, 2017). The defined number of PSCs and the culture medium employed led to an asymmetric structure containing epiblast-like cells and cells resembling the amniotic ectoderm. This structure remained stable even as the cells divided. This model represented the first platform to study human early development *in vitro*. In a further study, Simunovic *et al.* (2019) used micropatterned systems in a 3D setting to generate an *in vitro* 3D model of the human pre-gastrulation epiblast. The epiblast-like structure was shown to spontaneously break symmetry under a uniform dose of BMP4, further expressing early markers of the PS and EMT, suggesting initiation of gastrulation (Simunovic *et al.*, 2019).

Building on the PASE model, Zheng *et al.* (2019) further improved efficiency and reproducibility by using microfluidics to achieve a controllable system for recapitulating events involved in epiblast and amniotic ectoderm development. The hPSCs adopted a 3D organization that mimicked the formation of the amniotic sac. Remarkably, by modifying the culture conditions, the authors were able to induce axis formation and gastrulation-like events, observing cell populations that resembled PS-like cells and endoderm-like cells, as well as human primordial germ cell-like cells. This model is one of the first systems to capture the complexity of spatial relationships and cellular interactions during early post-implantation development and gastrulation in human (Clark, 2019).

Modelling gastrulation towards early organogenesis in mouse: embryo bodies and gastruloids

To date, gastrulation has been mapped in several model organisms, such as mouse, allowing the fate and location of specific cell types to be characterized (Vogt, 1929; Hatada and Stern, 1994; Tam and Behringer, 1997; Alev *et al.*, 2010). However, no such descriptions have been made in human, primarily due to ethical constraints on embryo culture (14-day rule). Yet a map of the gastrulating human embryo would be of immense value for providing more comprehensive insights into human development and allowing for species-specific differences to be evaluated. Moreover, a greater understanding of cell fate specification would inherently contribute to PSC differentiation efforts.

In a developmental context, embryoid bodies (EBs) have long served as a standard tool to assess PSC pluripotency, as aggregates of PSCs are able to differentiate spontaneously into the three germ layers (Table I) (Desbaillets *et al.*, 2000; Itskovitz-Eldor *et al.*, 2000). However, spontaneous differentiation of PSCs into EBs usually results in heterogeneous cell populations and terminally differentiated cells are rarely obtained, primarily due to the lack of control of each differentiation step. Nevertheless, in specific culture conditions, mouse EBs have been used to generate ELS that mimic symmetry breaking events observed during early post-implantation development (ten Berge *et al.*, 2008; Fuchs *et al.*, 2012). Modified culture protocols have enabled key aspects of post-implantation mouse development to be captured *in vitro* (Marikawa *et al.*, 2009; van den Brink *et al.*, 2014; Turner *et al.*, 2017; Beccari *et al.*, 2018). Analysis of these ELS using immunofluorescence and at the single-cell level revealed that they formed the three germ layers with reference to the three body axes (Beccari *et al.*, 2018; van den Brink *et al.*, 2020). As these processes are the consequence of gastrulation, the structures were termed gastruloids. Beccari *et al.* (2018) revealed that mouse gastruloids mimic the spatial and temporal patterns of *Hox* gene expression that determines the antero-posterior organization of the embryo. Mouse gastruloids can be used to study gastrulation, body axis establishment and the early phases of organogenesis in an animal-free and high-throughput manner *in vitro*. However, they do not generate anterior neural (brain) cells or any extra-embryonic tissues, and are not able to implant in utero, ultimately lacking full organismal potential. Although specific culture conditions can induce differentiation of gastrula organizer cell in hESC-derived EBs (Sharon *et al.*, 2011), no human version of these 3D-gastruloids has been reported thus far.

Limitations and challenges of ELS

PSC-based ELS represent an important addition to the human stem cell toolbox (Clark, 2019). However, these structures cannot fully develop and progress into viable embryos. ELS primarily lack critical cell types and cellular organization, leading to defective development. Furthermore, current ELS remain largely limited to the mouse, and the extent to which they recapitulate the transcriptomic and epigenetic signature of both *in vitro* and *in vivo* fertilized embryos is yet to be elucidated. Moreover, the crosstalk at the embryo-maternal interface that actively assists in the final stages of pre- and early post-implantation development (Gellersen and Brosens, 2014) is ultimately abnormal between ELS and the maternal uterus. As seen in mouse ELS studies, this leads to developmental arrest a few days after implantation. The complex yet crucial signals resulting from the interactions between the embryo and the surrounding maternal environment are yet to be uncovered and reliably captured *in vitro*. To date, the blastocyst is the only stage compatible with implantation, while proper interactions with the maternal endometrium are an essential prerequisite for further development (Evans *et al.*, 2012; Koot *et al.*, 2012).

Concurrent to scientific innovation, considering the extent to which the use of ELS raises ethical challenges related to human embryo research remains paramount. This will be crucial for harnessing the potential of ELS as a valuable research tool, whilst also remaining within a robust moral and legal framework of professionally acceptable practices. Ultimately, the extent to which ELS actually mimic fertilization-based embryos will govern the nature of the ethical and regulatory issues that stem from their creation and use (Pera, 2017).

Relevant ethical and policy aspects of the human embryo research debate

The increased opportunity of studying early human life in a dish ever since the advancement of IVF did not only hold great scientific and therapeutic promise, but it also raised a series of challenges about instrumental and destructive human embryo research (Mulkay, 1994). The public was 'divided between pride in the technological achievement, pleasure at the new-found means to relieve, at least for some, the unhappiness of infertility, and unease at the apparently uncontrolled advance of science, bringing with it new possibilities for manipulating the early stages of human development' (Warnock, 1984). Robust moral and legal directives capable of enforcing professionally acceptable practices were requisite in addressing these concerns.

Conceptual debate: the concept of 'the embryo'

The stipulation of necessary and/or sufficient conditions for an entity to qualify as a human embryo was particularly crucial to this end: before knowing *how*, one must know *what*, to regulate. An interaction between the natural sciences and the humanities on how to (re)define the human embryo and its constituent criteria was therefore essential.

The rise and fall of the 'pre-embryo'

In the past, little thought had been given to an exact definition of the 'embryo'. It was obvious that the mammalian 'embryo' referred to the developing entity resulting from the *in vivo* fertilization of an oocyte by a spermatozoon. Embryologists also commonly used the term interchangeably with 'ovum' or 'conceptus'; terms derivative from studies with invertebrates and other lower animals meant to designate 'the totality of cells derived from the fertilized egg' (McLaren, 1986).

As knowledge of embryology progressed, it became clear that, in early mammalian embryogenesis, the cells derived from the fertilized oocyte commit to distinct fates. Whereas a vast majority of these cells will develop into extraembryonic structures, only a small part of the ICM will actually transform into the so-called 'embryo-proper' or 'definitive embryo' (McLaren, 1986). Prior to cellular differentiation, referring to the developing entities as 'embryos' thus seemed to be 'no more (maybe less) appropriate than to refer to them as placentae' (McLaren, 1987). Several alternative terms have since been put forth in an effort to represent morally relevant differences in (extra)embryonic fates, with the 'pre-embryo' becoming by far the most popular one in scientific and ethical literature.

For critics, however, the conceptual distinction between embryos and pre-embryos conveyed the impression that they were also normatively different, i.e. that the latter were morally inferior to the former. The critique that the term defined moral problems away, ultimately led to its gradual abandonment before the mid-1980s. With notable exceptions, such as Spain (Table I), most other jurisdictions have since returned to 'embryo' as the designated term to denote the beginnings of early (human) life. Nevertheless, it remained necessary to specify the exact criteria the term conveys. This was clearly no easy task, as the conditions for embryo qualifications continue to vary considerably across jurisdictions (European Group on Ethics in Science and New Technologies, 1999).

Conditions for embryo conceptualizations: fertilization and potential

In the past, fertilization was, and in some jurisdictions still is,—presumed to be a necessary and sufficient condition for entities to qualify as embryos. The *Human Fertilization and Embryology (HFE) Act 1990*, for instance, referred to the embryo as 'a live human embryo where fertilization is complete' (Human Fertilization and Embryology Act, 1990) (Table I). Another example is the presently enforced Spanish *Law 14/2007*, which still defines the embryo as the 'fertilized oocyte' (Law 14/2007, of July 3th, on Biomedical Research, 2011) (Table I). Interestingly, while both examples refer to the same phenomenon as a point of reference for embryo conceptualizations, they are discrepant with regard to the intended phase of development. While the HFE Act demarcates the two-cell stage as the completion of fertilization, the Spanish Act fails to specify an exact phase, alluding to fertilization instead as a single, isolated 'moment' in embryogenesis. This variance can have important implications for regulation. Rather than being defined as a moment, fertilization is more appropriately understood as a process; i.e. a series of consecutive events in the 20–22 h following the meeting of an oocyte and a sperm, which include, among others, syngamy and zygotic genome activation (National Health and Medical Research Council, 2006). What, then, is the fundamental event in fertilization from which to begin counting? Fertilization-based embryo conceptualizations, if unspecified, prove too ambiguous for suitable, clear-cut directives.

Since Dolly's birth, the cloned sheep, also implied the possibility of producing offspring through SCNT in humans (Table I), it was widely felt that fertilization could no longer be regarded as a *necessary* condition for embryo qualifications (Health Council of the Netherlands, 2005; Piotrowska, 2019). Countries that have maintained it while also ratifying the Oviedo Convention (which forbids creating human embryos for research (Council of Europe, 1997)), make themselves vulnerable to charges of duplicity, allowing them to appear supportive of embryo protection while giving free reign to SCNT-research (Beriain, 2014; Dondorp and de Wert, 2017). In most jurisdictions, post-Dolly definitions of the human embryo have thus either come to refer to fertilization as a sufficient condition amongst others or substituted it altogether. An example of the former is Australian legislation, which widened the definition to include entities deriving from 'any other process that initiates organized development' (National Health and Medical Research Council, 2006) (Table I). Whereas the Australian definition still contains an implicit reference to fertilization, any such reference is abandoned in Dutch and Belgian legislation, which define the human embryo as 'a cell or cluster of cells with the potential to develop into a human being' (Embryos Act, 2002; Medically Assisted Reproduction and the Disposition of Supernumerary Embryos and Gametes Act, 2003) (Table I). Moreover, whereas the Australian definition looks at *commencing* development (limited to the first eight weeks of development since the first mitotic division), the Dutch and Belgian definition focuses on its *completion*.

Dutch and Belgian regulators do not further specify the exact meaning of 'the potential to develop into a human being'. Commentators have pointed out that, when this is understood as stretching unto birth, it legally implies that the concept of non-viable embryos is a *contradictio in terminis* (de Wert and Mummery, 2003). Similar disputes have emerged in two rulings of the Court of Justice of the European Union (Judgement of 18 October, 2011; Judgement of 18 December, 2014). In the *Brüstle vs Greenpeace* case, the CJEU ruled that organisms 'capable of commencing the process of development of a human being' (Judgement of 18 October, 2011) were to be regarded as human embryos for the purpose of the relevant EU-Directive. This would apply to fertilized oocytes, oocytes activated through parthenogenesis, and oocytes into which the nucleus of a mature human cell was transplanted (SCNT). When the relevant criterion was challenged with regard to parthenotes in the *International Stem Cell Corporation* case, the CJEU requalified its earlier ruling, stipulating that 'in order to be classified as a 'human embryo', a non-fertilized human oocyte must necessarily have the inherent capacity of developing into a human being' (Judgement of 18 December, 2014).

At stake here, is that each criterion (fertilization, commencing development or the potential to develop into a human being) differentially determines the scope for the applicability of embryo protective regulations and related normative debates. For instance, in the Dutch debate, it has been argued that the legal definition may be both too narrow and too wide (Winter *et al.*, 2012). Too narrow because it preempts the question of whether non-viable human embryos might also deserve some level of protection. Too wide because gametes and, with present technologies, even somatic cells, may also be said to have the potential to develop into a human being. Whether this implication can be avoided by the added qualification 'inherent' (as in the last quoted CJEU ruling) is a matter for debate, to which we return below.

Normative debate: the moral standing of early human life

In the words of Mary A. Warren, to have moral status is. . .

> '. . . to be an entity towards which moral agents have, or can have moral obligations. If an entity has moral status, then we may not treat it in just any way we please; we are morally obliged to give weight in our deliberations to its needs, interests or well-being. Furthermore, we are morally obliged to do this not merely because protecting it may benefit ourselves or other persons, but because its needs have moral importance in their own right' (Warren, 1997).

Sentient animals have moral status in this sense because, as entities capable of feeling pain and experiencing discomfort, they have interests that can be thwarted. Although the same goes for human beings, it is generally only with reference to them that the term 'full moral status' is used.

The idea is that there is more to humans than the features they share with (other) sentient animals. Of course, there are different interpretations of what this 'more' entails, ranging from the belief that human beings are created in God's image to the claim that humans are *persons*, i.e. that they have certain properties (such as self-consciousness, rationality and the capacity for intentional action) that command respect because they are essential for moral agency (DeGrazia, 2012). Thus, whereas the instrumental use of animals may, under conditions of proportionality and subsidiarity (Table I), be reconcilable with acknowledging their moral standing, it is generally argued that, given their *full* moral status, human beings should never be treated as mere means (Kant, 1997).

Now, what about the moral status of human embryos? Do they qualify as entities deserving of protection in their own right? If so, what level of protection do they deserve and what does that imply for their research use? This discourse has traditionally focused on the status of preimplantation embryos due to the former inability of culturing human embryos *in vitro* for longer than a few days. Here, three broad views can be discerned along a spectrum (Robertson, 1986). At one end is the view that, at preimplantation stages of development, the embryo is morally equivalent to a mere cluster of cells. This view 'allows the embryo to be treated like any other human tissue used in research, subject only to rules to protect the interests of those who have dispositional control of the embryo' (Robertson, 1986). At the other end are those who view preimplantation embryos as deserving the same moral reverence due to any human being (Ford, 1988). The rationale for this position, which is generally grounded in Christian beliefs, is that what makes human beings morally special also applies to human embryos from the earliest stages of development. Since full moral standing involves full protection, the resultant policy is to ban embryo research altogether, except for research aimed at benefiting the embryo itself. Between these extremes, the dominant view regards 'the embryo as deserving of respect greater than that accorded to tissue, but not as much as that accorded to persons' (Robertson, 1986). This view is often understood as gradualist, according human embryos an initially low but increasing moral status with certain developmental milestones (National Institutes of Health, 1994; Hermeren, 1996). As such, it is tolerant of embryo research, albeit under conditions of proportionality and subsidiarity.

The question is, of course, on what basis the third view accords this relative moral status to preimplantation embryos. Bearing in mind that the properties generally regarded as morally significant in the partly overlapping abortion debate, e.g. heartbeat, sentience or incipient brain activity (Tauer, 1997), have not yet emerged at this stage of development, the most promising feature appears to be their potential to grow into a human being.

The argument from potential

Ascriptions of moral status based on the embryo's potential to develop into a human being may depart from very different understandings of potentiality (Buckle, 1990). Those reasoning along the lines of classical Aristotelian teleology will understand this notion as 'active' or 'inherent' potentiality, i.e. as the organism's *intrinsic power* to become what it is destined to be. Assuming that the embryo is destined to become a fully developed human being, and given that (as persons) human beings are morally worthy ends, this active potential is what entitles it to moral standing. However, depending on whether the emphasis lies on the continuity or the difference aspect of the potential to self-realization, this may still lead to very different conclusions. Those emphasizing continuity, i.e. the view that the embryo is already the human person that it will become, will maintain that the fertilized oocyte has the same (full) moral status as any of us and may not be used as research material. Those emphasizing the still large difference between what the embryo is and what it will become, will accord it a much more limited moral status, allowing its instrumental use under certain conditions.

The embryo's potential to develop into a human being may alternatively be understood as 'passive' or 'contingent': as merely one of many possible outcomes, all of which ultimately rest on external events or actors. Those reasoning from this perspective point out that a fertilized oocyte has actually a greater chance of perishing than of it ever becoming a human being, thus making it difficult to take intrinsic-potentiality arguments seriously. Furthermore, if the fertilized oocyte has moral status by dint of its intrinsic potential to become a human being, then should not the oocyte and sperm, which combined have the same potential, be conferred equivalent moral standing? (Kuhse and Singer, 1982).

However, this *reductio ad absurdum* misunderstands the meaning of active potentiality. Given that this notion refers to 'the power possessed by an entity to undergo changes [. . .] to *itself*' (Buckle, 1990), it requires the preservation of individual identity throughout its actualization. Entities can therefore only possess the morally relevant potential from the moment they can be identified as numerically identical to the human being they will become. This impedes the ascription of active potentiality not only to gametes, but also to fertilized oocytes and pre-implantation embryos since, at these 'pre-embryo' stages, the cells from which the embryo-proper develops are still indistinguishable from those that will take on extraembryonic functions. As a consequence, it is only from the development of the embryo-proper onwards, which is also the stage from which natural twinning can no longer occur (Table I) that embryos can be said to possess the potential required for moral standing. While this reasoning may save active potentiality from the *reductio*-charge, it comes at the price of limiting its applicability to post-implantation embryos. At earlier stages, only passive potential can then be ascribed.

If prior to the emergence of the embryo-proper the argument from potential can indeed only refer to passive potentiality, this need not mean that there are no grounds for ascribing moral status to preimplantation embryos. Doing so, however, must then be a matter of granting early human life a certain degree of *symbolic value* or moral standing *by association*, rather than acknowledging any intrinsic moral value.

Conditions for acceptable embryo research

The so-called 14-day rule limits human embryo research to 14 days of development or equivalent developmental stages, such as the beginning of gastrulation or the formation of the PS (Warnock, 1984; Appleby and Bredenoord, 2018).

The specific stipulation of this limit ensued from moral deliberation on the biological qualities of early human embryos. Of special relevance were considerations regarding the beginnings of neurulation (Table I), with which sentience is associated,—and ontological individuation. Whereas neurulation occurs between 17 and 26 days after fertilization, natural twinning can still occur until 14–15 days after fertilization (Table I) (Warnock, 1984). Hence, in an effort to err on the side of caution, legislators limited embryo research to stages amply preceding the development of morally concerning features.

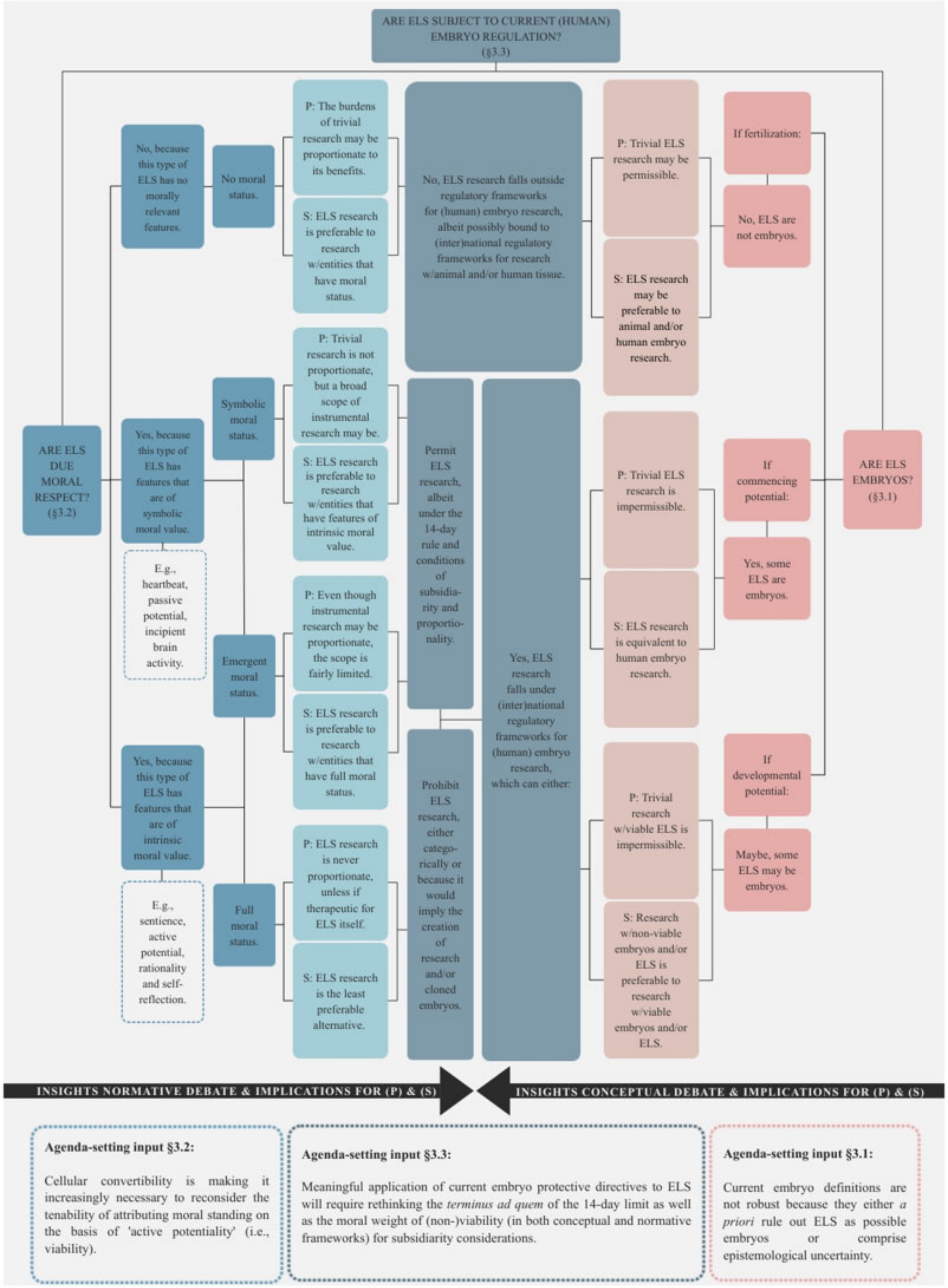
The precise reasons for keeping clear of these specific features were never spelled out. As ontological individuation establishes numerical identity between the embryo and the human individual it may grow into, reaching the stage where twinning is no longer possible seems relevant only for those subscribing to the idea of active potentiality. In fact, only for those linking this idea with the ascription of full moral status, would it seem obvious that ontological individuation poses an absolute limit to the moral acceptability of human embryo research. Equally underdetermined are the reasons for steering free from other features of concern. Sentience as such, for instance, is not a sufficient reason for a strict limit, given that research with sentient animals is accepted, albeit conditionally. Moreover, to the extent that features such as the beginnings of neurulation can be understood as relevant milestones in a gradualist account of moral status (whether intrinsic or symbolic), it is also not obvious why this would require categorically cutting-off research at day 14. The 14-day rule was thus not adopted because there were more convincing reasons for drawing the line here rather than at some later stage, but because it provided a pragmatic means to allay public anxiety while delineating a clear-cut and enforceable boundary (Warnock, 1984, 2007; Cavaliere, 2017; Warnock, 2017).

Despite its widespread and longstanding acceptance, scholars are now appealing for a reevaluation of the rule. At the time of its formulation, the possibility of culturing human embryos *in vitro* for longer than 14 days seemed too far-remote to be taken into consideration. At present, however, it is becoming a feasible reality (Hyun *et al.*, 2016), with scientists having managed to maintain human embryos alive for an unprecedented period of 13 days *in vitro*, after which they had to cut off their experiments (Deglincerti *et al.*, 2016a).

For embryo research to be permissible within this or any alternative timeframe, it must meet the additional conditions of proportionality and subsidiarity (Table I). The proportionality condition holds that embryo research must serve a morally important goal (Pennings and van Steirteghem, 2004). For instance, while the instrumental use of human embryos for the treatment of major diseases is acceptable, their use for the safety testing of cosmetics is not. Subsidiarity requires that no morally less problematic alternatives for reaching the same goal are available (Pennings and van Steirteghem, 2004). For instance, human embryos should not be used for research that can be done with non-embryonic cells or tissues, nor should they be created as research materials for studies that can be conducted with supernumerary IVF embryos. While it has also often been taken for granted that this principle favours the use of animals over human embryos, this is less obvious if human embryos have at best a relatively low moral status (Jans *et al.*, 2018).

Discussion

The importance of the scientific developments summarized in this review lies in the double expectation that human ELS may provide accurate means to study embryonic processes, while being sufficiently different from fertilization-based embryos to keep free from restrictions limiting their research use (Fig. 2). There is an obvious tension between these two perspectives that needs further exploration: to what extent can these models be applied, or improved upon, without raising (part of) the concerns behind the barriers that the modelling hoped to circumvent? Despite recent efforts towards the development of tangible guidelines (Hyun *et al.*, 2020;



Agenda-setting input §3.2:

Cellular convertibility is making it increasingly necessary to reconsider the tenability of attributing moral standing on the basis of 'active potentiality' (i.e., viability).

Agenda-setting input §3.3:

Meaningful application of current embryo protective directives to ELS will require rethinking the *terminus ad quem* of the 14-day limit as well as the moral weight of (non-)viability (in both conceptual and normative frameworks) for subsidiarity considerations.

Agenda-setting input §3.1:

Current embryo definitions are not robust because they either *a priori* rule out ELS as possible embryos or comprise epistemological uncertainty.

Figure 2. Overview of ethically relevant aspects in the traditional human embryo debate and respective implications for ELS research. Prominent conceptual and normative conditions for the qualification, moral standing and regulation of research with early human life in the traditional human embryo research debate are summarized and extended to (research with) ELS. Subsequent implications for the principles of proportionality (P) and subsidiarity (S) are shown.

ISSCR Statement on Ethical Standards for Stem Cell-based Embryo Models, 2020), the ethical and regulatory ramifications of this emerging field remain to be thoroughly defined. To this end, the present section juxtaposes the conceptual distinctions and normative positions previously discussed in the context of traditional human embryo research to (specific types of) ELS. Each subsection will focus on elucidating the ensuing conceptual, normative and regulatory implications for ELS (research), respectively.

Are ELS embryos?

As previously shown, there is no universally accepted biological, legal or ethical definition of the human embryo. This means there are different possible answers as to whether (certain) ELS qualify as human embryos. In jurisdictions where fertilization is a necessary condition for such qualification, ELS are clearly not embryos as none of these constructs arise from the fusion of gametes.

If, as in alternative definitions, it suffices that the relevant entity has the *potential* for embryo-like development, it becomes a matter for debate whether certain ELS qualify as such (Piotrowska, 2019). In Australia, for instance, PASE, gastruloids and ETS/X constructs might, whereas blastoids might not (Table I). If the definition requires *the potential to develop into a human being*, as defined by Dutch and Belgian legislation, it becomes unclear how to categorize ELS (Hyun *et al.*, 2020). The fact that present-day ELS develop only up to a certain point implies that they cannot (yet) qualify as embryos under this definition. Moreover, defining the embryo in these terms presents an epistemological challenge in that we cannot know whether the definition applies to ELS without conducting experiments that would themselves raise ethical concerns. Would it suffice if animal experimentation were to suggest that improved ELS could lead to offspring in at least other mammals? Similar post-Dolly reasoning seemed to be the basis for regarding SCNT-products as embryos under Dutch and Belgian legislation (Health Council of the Netherlands, 2005). Then again, the potential to develop into a human being may be 'switched off', for instance, by knocking out the genes necessary for development beyond a certain stage. As in the earlier SCNT-debate, where it was proposed to use modified stem cells so as to circumvent US public funding rules (Grompe, 2005), the ability to switch certain features 'on' and 'off' might be seen as providing a way to ensure that, at least in terms of such-like definitions, ELS can never qualify as embryos (Pera *et al.*, 2015).

Importantly, the question whether ELS qualify as embryos is not just relevant for scientists wanting to determine the ethical and regulatory boundaries of their work, but also the other way around; our analysis shows there is an urgent need for policymakers to reconsider the reasoning behind present definitions in light of current developments. Whereas fertilization-based definitions are clearly too narrow, as they preempt the question whether human beings could develop from processes other than the fusion of gametes, potentiality-based definitions may be too wide, depending on how the notion of 'potential' is understood.

While legislators may have intended 'the potential to develop into a human being' to mean active potentiality, it has been argued that this concept is no longer tenable in view of recent technological advancements (Stier and Schoene-Seifert, 2013). In particular, these advancements do not only show how very different types of human cells may be converted into 'baby-precursors', but they also emphasize the extent to which, even in standard human reproduction, embryo development is dependent upon 'innumerable external biochemical triggers' (Stier and Schoene-Seifert, 2013). In this sense, there appears to be no difference between the potential of a skin cell, a pluripotent stem cell, or a zygote: with the right kind of external triggers, each can be made to develop into a human being. Although this remains a contested position (Cunningham, 2013; Hyun, 2013), the very debate suggests that, if unspecified, the notion of *developmental* potential does not provide a solid basis for distinguishing between embryos and non-embryos. Moreover, definitions in terms of this notion also appear to imply that, for the purposes of regulation, there are no such things as non-viable embryos.

Definitions in terms of *commencing* potential avoid these challenges while remaining conscientious of what scientific practice regards as embryos. On the other hand, the fact that this definition would include ELS that are structurally incapable of developing into a human being, will be regarded by many as casting the definitional net too wide. Of course, the question of whether ELS are to be regarded as embryos should be distinguished from the further question of whether and to what extent they deserve protection. If non-viability does not inhibit ELS from qualifying as embryos, it may still be a good enough reason for according them a lower moral status, thereby allowing more room for their use in research.

Are ELS due moral respect?

First, to say that (certain) human ELS are not embryos does not imply that they cannot have moral status or that their development cannot raise moral concerns. For instance, should it become possible to develop ELS capable of feeling pain, then, this ability alone grants them a moral standing akin to that of sentient animals. Moreover, regardless of whether or not ELS qualify as human embryos, they may still develop features that many would consider morally concerning, such as incipient brain activity or an emerging human form. Similar issues are found in the context of research with cerebral organoids (Farahany *et al.*, 2018; Lavazza and Massimini, 2018; Hostiuc *et al.*, 2019).

Second, if (certain) ELS are embryos, then, depending on the definition underlying this qualification, they either do or do not have the potential to develop into a human being. Short of becoming sentient, ELS lacking this potential cannot be conferred more than symbolic worth. Conversely and hypothetically speaking, if ELS were to have this potential, the question becomes whether it is understood as *passive* or *active*. *Passive* potentiality would imply that ELS could at best have moral value *by association*: they owe their moral status to the symbolism associated with what they represent, i.e., the beginnings of early human life. This value may then increase with the achievement of developmental milestones. *Active* potentiality would imply that ELS have intrinsic value and, therefore, independent moral status. Here, views diverge. For some, ELS with active potential would be due full moral standing, regardless of their developmental stage. For the majority, however, while active potential would confer ELS independent moral status, this status would be initially low and gradually increase with further development.

Moreover, for those who accept the view that active potentiality requires ontological individuation, i.e. that the relevant entity must be the same organism (in terms of numerical identity) as the human being it will develop into, it follows that only ELS capable of modelling post-implantation development (e.g. the 'embryo-proper') could have this potential and respective (intrinsic) moral status. In this view, both ELS limited to modelling stages preceding individuation (such as blastoids), and fertilization (or SCNT)-based embryos at corresponding stages, would thus have symbolic worth at most.

ELS as a policy challenge

If (certain) ELS do not qualify as embryos, it follows that their research use will not be subject to regulations any more than is the case for research with (human) cells and tissues generally. Aside from the fact that this may enable the study of developmental stages for which, given the widespread acceptance of the 14-day rule, human embryos cannot be used, it may also imply that research with ELS ought to be prioritized over research with animals or human embryos from a subsidiarity perspective. Nevertheless, given the possibility of ELS raising moral concerns equivalent to those raised by animal (e.g. sentience) or post-implantation human embryo (e.g. heartbeat) research, there may be a regulatory lacuna here.

If (certain) ELS are embryos, the question arises how current embryo research regulations apply to them. Research with ELS that qualify as such will not only be prohibited in jurisdictions that forbid embryo research altogether (e.g. Germany (Embryo Protection Act, 1990)) but also in jurisdictions where the creation of embryos for research purposes is not allowed (e.g. all countries that ratified the Oviedo Convention). Some may view this as a further occasion for questioning the 'discarded-created' distinction behind this ban (Devolder, 2005), or point to the fact that the 'feminist' argument against creating embryos for research (referring to the burdens and risks of oocyte donation (George, 2007)) does not apply to the creation of ELS.

A further issue is how ELS research relates to cloning. Depending on whether hESCs or iPSCs cells are used, ELS research may lead to entities that are a genetic copy of either the embryo from which hESCs were derived (i.e. 'embryo cloning') or the individual whose cells were used to create iPSCs (i.e. 'adult cloning'). It is important to keep this in mind because, even though most countries only forbid human *reproductive* cloning, some also prohibit human cloning for research purposes (Isasi and Knoppers, 2006; Paolo Busardò *et al.*, 2014), thereby impeding both SCNT and ELS research.

Another prominent and currently widely debated issue is the elusiveness of applying the 14-day rule to research with entities whose development starts at what for fertilization (or SCNT)-based embryos would be different post-fertilization stages (Hyun *et al.*, 2020). A meaningful application of the rule to ELS would thus require its *terminus ad quem* to refer to morally relevant features rather than the mere duration of development (Aach *et al.*, 2017). In this respect, the explicit reference to the appearance of the PS as an alternative ground for limiting embryo research whenever this would come before reaching the time limit, seems to give the British *HFE Act* an advantage vis-à-vis articulations only referring to the first 14 days, as the Dutch and Belgian Embryos Acts. In any case, pragmatic arguments for maintaining the 14-day limit must give way to the inevitability of reconsidering the material grounds for regarding the appearance of the PS, or any other subsequent developmental feature, as a point after which human embryo research would be morally unacceptable. The fact that PS formation is a core developmental feature of certain ELS underscores the urgency of this debate. Note that this is not just a matter of the traditional framework falling short of new developments, but also of those developments (further) revealing the indeterminateness of the reasoning behind the 14-day limit as a cornerstone of the traditional framework.

The same is true regarding the requirement of subsidiarity: ELS showcase how current frameworks lack specific regulations for non-viable embryos. Again, depending on the exact definition, non-viable embryos either fall outside the scope of embryo research regulations, or are subject to the same regulations as viable embryos, where neither of them is given priority if both can be used as research material. There may, however, be good reasons for prioritizing non-viable embryos as research material, for instance, because any moral worth accorded to them can at most be symbolic, whereas viable embryos may be understood as having an intrinsic value based on their presumed (active) potential. Without such prioritization, the standard application of the subsidiarity principle would impede the creation of embryos (including the creation of ELS that qualify as such) for research purposes that can be pursued with supernumerary IVF embryos. For those who argue that (non-) viability is merely contingent upon the availability of the right external triggers, the case for prioritizing research with non-viable embryos (and ELS) is weakened. Here, the development of (the currently non-viable) ELS reveals again the indeterminateness of present normative frameworks at a crucial point.

Conclusion

PSC-based ELS recapitulate various aspects of early mouse and human development, including the formation of the epiblast, trophoblast and amniotic cavity, as well as gastrulation-like events. Overall, ELS remain a promising platform for elucidating critical processes during mammalian embryogenesis, potentially delivering greater flexibility and higher-throughput compared to studies involving fertilization-based embryos. Given these benefits, it is reasonable to expect that the quality of ELS will rapidly improve, allowing experts to mimic aspects of embryogenesis ever more accurately. Where human ELS are concerned, this may prove especially favourable in evading the ethical and legal restraints imposed on the use and/or creation of human fertilization-based embryos for research. At the same time, the more ELS succeed in replicating natural development, the more urgent it becomes to consider the extent to which their use may raise (part of the) moral concerns typical of human embryo research. In terms of agenda setting for ethical refection and societal debate, the following issues stand out.

A first issue concerns the robustness of present embryo definitions underlying embryo protection regulations. To be adequate, such definitions should not *a priori* rule out the possibility of ELS qualifying as human embryos, nor should their applicability comprise epistemological uncertainty. In this regard, defining the embryo in terms of its ability to initiate embryogenesis, thus remaining closest to what scientific practice regards as embryos, seems the more promising alternative because it avoids limiting the scope for moral debate on a conceptual level.

Secondly, it needs to be clarified whether the concept of active potentiality can survive as a basis for according moral standing in times where embryos can be assembled and disassembled as cellular building kits. If the concept must be abandoned also for fertilization (or SCNT)-based embryos, present normative frameworks for embryo protection would require much rethinking. However, if active potentiality remains an important cornerstone of the framework, programmed non-viability would keep ELS, and fertilization (or SCNT)-based embryos subjected to similar programming, in a different moral league, where symbolic rather than intrinsic value is all that possibly counts. This is directly important for determining whether such ELS (as well as non-viable embryos generally) should be given priority as research material under the principle of subsidiarity.

A final issue urgently requiring ethical reflection and debate is the 14-day limit as *terminus ad quem* of human embryo research. Where understood as referring to mere developmental duration, the rule cannot be meaningfully applied to ELS because their developmental stage at the first day of culture can correspond to that of a several days old fertilization (or SCNT)-based embryo. Instead of a time limit, what is needed is an account of morally relevant features, the emergence of which would render research with human embryos (even when non-viable) problematic. Whether PS formation would qualify as such, is at least not obvious. Here again, what complicates the necessary debate is the developmental fluidity of ELS, not only allowing the bypass of certain stages all together but also enabling certain features to be switched 'on' and 'off'.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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