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Embryoids, models, embryos? We need to take a new look at legal norms concerning the beginning of organismic development

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Introduction

The recent progress in research on embryo-like structures (ELS) derived *in vitro* from pluripotent stem cells is impressive (Amadei *et al.*, 2022; Rossant and Tam, 2022; Tarazi *et al.*, 2022; Abel and Sozen, 2023; Apostolou *et al.*, 2023; De Santis *et al.*, 2023; Handford *et al.*, 2023; Karvas *et al.*, 2023; Nakatani and Torres-Padilla, 2023; Oh *et al.*, 2023; Oldak *et al.*, 2023; Pedroza *et al.*, 2023; Tam *et al.*, 2023; Weatherbee *et al.*, 2023; Yu *et al.*, 2023). Such constructs have been shown to reach even postimplantation stages, since these investigations can take advantage of methodological progress in culturing early mammalian embryos *in vitro*. Methods of prolonged culture now allow also for a more stringent testing of the potentialities of stem cell constructs (Bedzhov *et al.*, 2014; Bedzhov and Zernicka-Goetz, 2014; Deglincerti *et al.*, 2016; Shahbazi *et al.*, 2016, 2019; Posfai *et al.*, 2021; Yuan *et al.*, 2023). Studies on embryology and those focusing on implantation mechanisms are mutually profiting from combining these methodologies (Tian *et al.*, 2022; David *et al.*, 2023).

All this progress is now accompanied by much attention from a broader public, and it is good to see that this is so. An important aspect that is increasingly realized is that these developments must stimulate us to *re-contemplate the term 'embryo'*. This is urgently needed because the definition of this term is critical for ethical considerations and legal regulations of research, since terms such as 'embryoid', 'model', and many others in use imply connotations about potentiality and viability (Blasimme and Sugarman, 2023; Iltis *et al.*, 2023). However, much confusion has arisen owing to the recent trend to use a wealth of different terms for the various embryo-like constructs produced using stem cells and, in particular, by introducing the term 'model' for them generally. Previous definitions for the term 'embryo' have been based on the traditional modes of generation by fertilization (no matter whether *in vivo* or *in vitro*) but are clearly not comprehensive enough anymore, since the recent studies amply demonstrate alternative routes (not involving oocytes and sperm) for the production of more or less complete ELS, and questions about principal viability of these constructs are now on stage. A recent publication by a number of prominent experts in the field (Rivron *et al.*, 2023) uses this viability aspect as the starting point for proposing a new ethical framework for human embryology with 'embryo models'; remarkably, these authors discuss reasons for applying the term 'embryo' (rather than 'model') to at least some of these stem cell-derived constructs. Principal viability of such human embryoid constructs must indeed raise ethical concerns if the constructs are produced for research purposes, an aspect which is now increasingly being realized (Rossant and Fu, 2023). The present article focuses on biological aspects of defining the potential to develop organismic wholeness (and thus viability) and underlines the functional aspects. It will be discussed how these aspects should be considered in updated definitions, focusing on the term 'embryo'.

The confusing recent use of terms

Most of the recent publications about stem cell-derived embryo-like constructs address these as '*models*', thereby following a proposition made by the International Society for Stem Cell Research (ISSCR) (Lovell-Badge *et al.*, 2021; Rossant and Tam, 2021). The term 'model' does indeed reflect the actual intentions of the researchers who create these constructs because these offer ample possibilities for studying elementary developmental mechanisms (such as cell differentiation into the various germ layer derivatives, pattern formation etc.), whereas this type of research has been problematic, and even impossible to perform on a large scale, in the past and in particular in the human (the mouse is not a perfect model for human development). The use of the term 'model' detracts attention, however, from implications of possible viability of the constructs. Previously, these constructs have been addressed generally as, for example, 'artificial' or 'synthetic embryos' (Denker, 2014), 'synthetic human embryos with embryo-like features, SHEEFs' (Aach *et al.*, 2017), or 'stembryos' (Veenvliet *et al.*, 2021), and many other terms have also been proposed (ELS (Pereira Daoud *et al.*, 2021), embryo-like assembloids (Ai *et al.*, 2023)). With an emphasis on the different degrees of completeness of the various types of construct, more specific terms have also been proposed (blastoids, polarized ELS, gastruloids, perigastruloids, post-implantation amniotic sac embryoids, iDiscoids etc.) (Shahbazi *et al.*, 2019; Hislop *et al.*, 2023; Liu *et al.*, 2023) (for an overview, see Table 3 in Iltis *et al.* (2023)). The ISSCR Guidelines distinguish between 'integrated stem cell-based embryo models', which possess derivatives of

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extraembryonic cell types (e.g. blastoids, ETX/iETX embryoids), and 'non-integrated stem cell-based embryo models' (e.g. gastruloids, Lovell-Badge *et al.*, 2021). I will address all of these stem cell-derived embryoid constructs here as '*embryoids*'.

The term '*model*', as used in the ISSCR Guidelines, is now indeed predominantly in use. This is very unsatisfactory, however, because when ethical implications of producing such constructs with human cells are to be contemplated, that term is at least imprecise and can be misleading: it suggests that aspects of possible viability of the 'models' do not need to be considered. In many publications that are dealing with this topic, we can already read the assumption that these 'models' are of course 'non-viable'. However, viability can only be checked in a stringent manner by transfer to a receptive uterus, and there is international agreement at present that such a viability test (corresponding to reproductive cloning) should not be carried out in the human, for ethical reasons. In this context, it must be of considerable interest that in recent experiments in a non-human primate model, the Cynomolgus monkey, blastoids have been shown to be able to implant in a receptive uterus *in vivo*, although development stopped at some point after implantation (Li *et al.*, 2023). In the human, blastoids have been reported to spontaneously form the first axis, and to attach directionally via polar trophoblast to hormonally stimulated endometrial cells *in vitro* (Kagawa *et al.*, 2022). Even incomplete human embryoids (lacking a trophoblastic shell, therefore not completely 'integrated' in the terminology of the ISSCR) show, under certain *in vitro* conditions, a very ordered anterior–posterior organization of the primitive streak, early neurulation and organogenesis (Liu *et al.*, 2023). Experiments in the mouse have revealed that development of stem cell-derived embryoids of various kinds can reach very advanced postgastrulation and neurulation stages (Amadei *et al.*, 2022; Lau *et al.*, 2022; Tarazi *et al.*, 2022).

Implantation and gastrulation (primitive streak formation) have previously been regarded by many as horizons in development, points at which a nascent individuum reaches crucially higher levels of organization on its way toward full viability, therefore representing steps that deserve to receive our highest attention under ethical considerations (c.f. the primitive streak/gastrulation stage in the Warnock Recommendations (Warnock, 1985, 2001) and the resulting UK regulations). The ISSCR Guidelines have already been criticized for a certain inconsistency with regard to implantation (an aspect of reproductive biology) and gastrulation (an embryological aspect): The Guidelines rule that 'stem cell-based embryoid models' should not be considered to be equivalent to human embryos and, therefore, they should not be subject to the same full set of restrictions which apply to natural embryos; however, while it is proposed that production of such 'models' should be acceptable, their subsequent transfer to a uterus should be strictly forbidden (irrespective of recipient species) (Boiani *et al.*, 2022). This seems to imply that the authors of those Guidelines do not exclude that some of the 'models' could deserve the same degree of respect and protection as traditional embryos, or at least they might in the future, as technological progress continues. This aspect is indeed the starting point for a recent critical analysis (Rivron *et al.*, 2023). Recently, even some of the authors of the ISSCR Guidelines are calling for caution when planning to produce and use the more complex embryoid 'models', in particular 'integrated models' (Rossant and Fu, 2023).

An aspect that is sometimes regarded important in ethical considerations is the degree of artificiality of the embryoid constructs, since the procedures needed for their production from stem cells appear complicated. Some of the procedures that have proven successful in the production of embryoids do indeed include, for example, the combination of defined numbers of cells of certain early embryonic stem cell lines (such as trophoblast stem cells and extraembryonic endoderm-like stem cells, in addition to naïve pluripotent or epiblast-like stem cells) in a certain physical arrangement (e.g. Harrison *et al.*, 2018; Ai *et al.*, 2023) or the introduction of a signaling center (Xu *et al.* 2021). Such experimental details seem to be helpful for establishing future embryonic axes (for a basic body plan) in a correct manner in a large number of constructs. This has sometimes been interpreted as showing that some type of 'embryo engineering' is required for the creation of embryoids, assuming this cannot occur without complicated manipulations. For some philosophers, a 'natural' origin adds to the degree of dignity we should ascribe to an entity, whereas 'artificiality' distracts from it. However, many of the recently published procedures require little detailed physical manipulation, and spontaneous budding of embryoids can be observed (even in large numbers) under certain conditions in dense cultures (discussed in Denker (2021)). On the other hand, most authors emphasize, in their description of embryoid production, the aspect of 'self-organization' (Pedroza *et al.*, 2023) (although this term obscures that the mechanisms behind are far from being understood in detail; for a discussion of molecular details of these processes, see Serrano Morales *et al.*, 2021). The recent advances in the field of stem cell-derived embryoids (i.e. technical tricks, such as the appropriate choice of culture media supplements, while gene overexpression in the cell lines used may be dispensible (Oldak *et al.*, 2023; Pedroza *et al.*, 2023; Weatherbee *et al.*, 2023)) suggest that indeed relatively little 'engineering' (artificiality) is required in order for self-organization to be initiated in colonies of early embryonic type stem cells (in particular naïve type stem cells, for example two-cell-like cells) if appropriate media supplements are provided (Denker, 2021). As far as the mechanistic background is concerned, it was discussed some time ago what developmental biology teaches us about the basic principles of how self-organization up to basic body plan development (and beyond) can be initiated in stem cell colonies. Such colonies appear to possess the ability to make use of minute inhomogeneities in culture as a substitute for factors initiating symmetry breaking and axes development, whereas in regular embryogenesis, the asymmetry cues are provided by the oocyte cytoplasm (Denker, 2004, 2020). There seems to be good reason to expect that further technical improvements might allow development of stem cell-derived embryoids to proceed much further than demonstrated so far. Specifically, blastoids (which can be produced in large numbers (Yu *et al.*, 2023) and can be transferred to a receptive uterus), if optimized according to further improved protocols, could acquire the potential for full development, as the already mentioned recent experiments in non-human primates (Li *et al.*, 2023) and observations on their *in vitro* development (Karvas *et al.*, 2023) suggest.

Toward an updated definition of the term 'embryo'

So how should we address stem cell-derived embryoids appropriately, in order to avoid any prejudgment that they are principally 'non-viable' (as at least a broader public so far seems to assume without any further questioning, when talking about the 'models')? We obviously need a more precise use of language. The aspects raised by recent embryoid 'model' research clearly

show that fertilization of an oocyte can no longer satisfactorily be seen as an obligatory starting point for organismic development in higher animals and the human (Matthews *et al.*, 2021; for aspects of the development of organismic wholeness see also Gilbert and Sarkar, 2000). To re-contemplate this fact now is certainly a pressing need, in particular for certain countries in which the term 'embryo' is of high juridical importance (e.g. in Germany (Bundesministerium der Justiz, 1990) (for a comparison of legislations, see Matthews and Morali, 2020) and supplementary data 2 in Rivron *et al.* (2023)). Even when considering other legislations, the definitions given in the ISSCR Guidelines can still be criticized for not being discriminative enough in addressing regulations concerning the various types of embryoid 'models' (Boiani *et al.*, 2022).

Novel definitions for the term 'embryo' are discussed in a recent article on an ethical framework for human embryology with embryo models (Rivron *et al.*, 2023). Remarkably, those authors present two different definitions: they start with a 'biological definition of an embryo', and then propose a 'legal definition of the embryo in light of embryo models', and these definitions differ considerably. The 'legal definition' is at odds with the 'biological' one, insofar as those authors include in the criteria to be fulfilled by an entity for being legally considered an embryo, uterine factors (which may be replaced *in vitro* by media supplements such as growth factors). From the biological point of view, the first part of the definitions (the 'biological definition') can easily be accepted. It is in agreement with the known facts and textbook definitions (embryo versus fetus depending on developmental stage/age, derivation of the embryo proper from the epiblast, extraembryonic cells, and appendages including trophoblast, chorion, yolk sac, amnion, allantois, placenta). The authors emphasize, in a later part of the text, the importance of functional aspects, specifically under the term 'potentiality', and to distinguish here between an *active* and a *passive potential*. Applying this active/passive potentiality concept (derived from Aristotelian philosophy) to questions of stem cell research (i.e. embryoid formation) had already been proposed in the context of aspects of autonomy in the development of a basic body plan (Denker, 2009). Rivron *et al.* (2023), however, refer to this distinction in a different sense: they propose to list uterine factors as an essential element for a legal definition of 'embryo', as follows (Fig. 2 in Rivron *et al.* (2023)):

> '***Biological definition of the human embryo:***
> *Human cells with the active potential to form a fetus.*
> ***Legal definition of the human embryo:***
> *Human cells with the active potential to form a fetus*
> *+ Support elements fulfilling extraembryonic functions*
> *+ Support elements fulfilling uterine functions.'*

I would suggest that their 'biological definition' is basically acceptable and sufficient. It is in agreement with biological facts and should, therefore, be a basis also for any legal norms, if completed by mentioning '(the potential to form) support elements fulfilling extraembryonic functions', i.e. as follows:

> ***Proposed biological (= legal) definition of the human embryo:***
> *Human cells with the active potential to form:*
> *- a fetus as well as*
> *- support elements fulfilling extraembryonic functions (i.e. extraembryonic cells and appendages like trophoblast, chorion, yolk sac, amnion, allantois, placenta).*

Such a definition would conform with the traditional definitions of terms like 'embryo proper' (the precursor of the fetus), 'extraembryonic appendages', and the combination of both, i.e. what is commonly addressed as the 'conceptus'. For linguistic reasons, it might be helpful to find a new term for 'conceptus' applicable to *in vitro* constructs with the same structures, with the reason being that these are not 'conceived' naturally by a woman. The 'Support elements fulfilling uterine functions' should be omitted from the definition for reasons I will discuss below. Any legal definition that differs from a biological one does not appear helpful and is potentially confusing!

Understanding embryoids as biological systems

I suggest that, from a biological point of view, when we attempt to re-define any entity with whose formation individual life may begin, we would have to do this in functional terms, i.e. in terms of systems theories applied to developmental biology. In doing so, we should focus strictly on what is known about morphogenetic mechanisms, pattern formation, and differentiation etc. Biological systems are often addressed as 'open systems', a viewpoint that is indeed of help when discussing how they react when responding to signals from their environment and when interacting with it (which they typically do). But biological systems are never completely open nor are they completely closed. In order to maintain their (bodily and functional) intactness, they must maintain aspects of closure. So, in case of disease, they react by specific regulations, i.e. up- or down-regulation of functions (fever), or repair activities (wound healing). If these regulations fail, the organisms die. In development, the germinating mammalian individual has a very impressive potential for regulation of structural defects, in particular in the early phases of development (twinning; the term 'individuation' is quite an unfortunate one in this context since it has led to much confusion, but we may have to live with its inadequacy since it is so much in common use, although imprecise (Denker, 2015)). Morphogenetic potential to develop into an organismic whole is, in higher animals (mammals), an intrinsic property that is already present in early embryos and does not depend on instructive signals from the environment (uterus).

Remarkably, trends in the ways of dealing with such systems-focused aspects have been changing in recent years (see also (Gilbert and Sarkar, 2000)). With regard to mammalian embryogenesis, an idea favored by some for many years was that the very early mammalian embryo (morula, blastocyst) could be seen as a system that is quite incomplete and widely 'open' in the sense that it would lack essential information about the development of structural order, in particular for the development of body axes (dorsoventral, anterior–posterior) and thus for a basic body plan. Starting in the 1960s, influenced by the then dominating *zeitgeist*, it was assumed, for example, that this information is provided by interaction with the uterus. At first, even the embryonic–abembryonic (future dorsoventral) axis of the blastocyst was proposed to be induced by some components provided by the uterine secretion: the term 'blastokinin' was explicitly chosen for a uterine protein to indicate a suspected specific blastocyst inducer function ((Krishnan and Daniel, 1967; Daniel, 2000); the protein was alternatively named uteroglobin); this assumption was not substantiated in the following years. Furthermore, until more recently, the anterior–posterior/craniocaudal axis of the embryo was thought by many to be determined by cell–cell interactions with the endometrium during implantation; this assumption was also unsubstantiated, at first owing to observations on continuing development after blockage of implantation *in vivo*, and more recently to studies on *in vitro*

developing embryos (discussed in Denker (2016)). From what we know now, we must conclude that the 'system early mammalian embryo' (e.g. already a morula) is functionally complete with regard to all specific morphogenetic information needed and is in this sense a closed system, autonomous with regard to its morphogenetic potential, no matter that development can be disturbed by noxious agents of various kinds. But, in the protective environment in which it normally develops (within the tube, the receptive uterus), it can express its program of differentiation and pattern formation in an autonomous way, and thus its totipotency. It does not depend on specific morphogenetic signaling from the reproductive tract. The question of what closure really means in mature biological systems and in developing ones is an important topic in the field of philosophy of organismic wholeness and developmental autonomy (Moreno and Mossio, 2015).

In terms of biological systems theories, and in order to refer to active potentiality, an embryo might, therefore, be defined as follows:

> *An embryo is a morphogenetically closed biological system capable of developing autonomously into an organismic whole under appropriate conditions (active totipotency).*

In the case of embryoid formation, the passive potential of (e.g. naïve type) pluripotent/omnipotent/totipotent stem cells can transform into an active totipotency after colony formation.

It must appear surprising that Rivron *et al.* (2023) now attach, in their 'legal definition' of the term 'embryo', a critical importance to uterine factors. Obviously, this must be understood in the way that these authors imply a constitutive (causative) morphogenetic role for these factors, i.e. that the latter factors would be much more than components necessary for intrauterine growth but would even exert a specific instructive, morphogenetic function, for which, however, we have no evidence.

Although I disagree with the proposition by Rivron *et al.* (2023) to declare uterine factors a constitutive element for a legal definition of the term 'embryo', I feel that their report has the general merit of directing our attention to the importance of functional aspects, specifically of developmental potentiality, when it comes to ethical considerations concerning embryoids. However, how can this be translated into practice? It is a difficult task to detect whether a colony of pluripotent stem cells (or any combination of it with other stem cells) has reached the state of a developmentally complete system (closed, autonomous in terms of developmental properties, active totipotency for organismic development initiated). The primitive streak state has been regarded until recently as a useful indicator, demonstrating morphologically that the entity in question has reached a state of incipient basic body plan development. As the developmental biologist knows from experimental experience, developing systems are always functioning before morphogenesis has yielded visible structures. Functionality of a developmental system precedes its morphological results. Functionality of the developmental system is, however, the basis for active potential of the developing entity. It remains a task for future investigations to find ways how to detect the functional completeness of the 'system early embryo', before the formation of a primitive streak demonstrates it morphologically. It has long been recognized that it would be desirable to initiate research projects aimed specifically at finding molecular and structural criteria that may be useful for this purpose, but such projects have not been funded so far (for the present state of knowledge about molecular characteristics, see Ishiuchi and Sakamoto, 2023). In order to avoid the ethical problem of possible viability of human embryoid constructs, more effort should be invested in comparative experiments with non-human primate models, which indeed have made progress recently (Bergmann *et al.*, 2022; Rodriguez-Polo and Behr, 2022; Li *et al.*, 2023).

As long as the functional completeness of the developing system cannot be detected more precisely on the basis of such criteria, how should we deal with the confusing wealth of terms presented in the literature for stem cell-derived embryoids and the lack of a clear relation to their developmental potential? Should we try to find a new but more general term than 'embryo', since 'embryoid' or 'model' seem to suggest non-viability? Terms proposed so far for addressing stem cell-derived constructs in general, such as 'stem cell-based embryo models', 'SHEEFS', or 'stembryos' (mentioned in the introductory remarks), have not received wide acceptance. With functionality in mind, a term like 'germ' could appear to be a candidate. In old German literature on developmental biology, the corresponding term 'Keim' has indeed been used more often, for example by Seidel (1960); it refers to both sexually and asexually (without oocytes and sperm) derived developing entities. However, tradition will most probably prevent us (in particular practitioners, for example in ART centers) from abandoning the term 'embryo'. Thus it might be wise to live with it and just to avoid specifying fertilization (combination of oocyte and sperm) as the exclusive origin.

Conclusion

The developmental potential of an entity (fertilization-derived embryo or stem cell-derived embryoid) should be seen as the ethically relevant biological property needing attention. It has been pointed out for a long time that the self-organization potential of stem cell colonies *in vitro* can lead to incipient individuation, and that its ethical aspects must be considered (Denker, 1999, 2004, 2006). As far as definitions are concerned that are suitable for legal norms, the above-mentioned wording for a 'Proposed biological (= legal) definition of the human embryo' could be a sufficient basis. Interestingly, other authors (focusing on oocyte-derived embryogenesis) have already pointed out some years ago that various techniques not involving sperm are available for producing viable embryos (such as somatic cell nuclear transfer, or parthenogenesis), and they have, for this reason, come up with a biological definition of the term 'embryo' (see below for details) (Findlay *et al.*, 2007). This has led to current legal regulations in Australia (Australian Government, 2006). I think it is very appropriate to reconsider this latter proposal in the light of stem cell-derived embryoids, because those definitions could still serve well for the future, if the wording is extended clearly enough to mentioning stem cell-derived embryoids. That proposition originally reads as follows (Findlay *et al.*, 2007) (and it was included, with nearly identical wording, in Australian law (Australian Government, 2006)):

> *'A human embryo is 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'.*

I propose that, as an adaptation to the new developments and for clarity, the second point of this definition [point (b)] could

simply be amended by explicitly adding, after 'genome', the following: '(e.g. stem cell-derived constructs)'. Any future legal regulations for research on advanced variants of embryoids that will now have to be discussed, should be built on a definition of this type, in agreement with established biological facts. Such definitions would indeed also conform with the wording of the Dickey-Wicker Amendment (US legislation, cited by Blasimme and Sugarman (2023)) since it lists not only gametes but also 'human diploid cells' as a possible origin of an embryo. Definitions along these lines would be fitting for stem cell-derived embryoids. However, as long as sufficient criteria for the detection of functional completeness of the system (active potential) are not available, warnings are in place to be restrictive with the production of human embryoids. I concur in this regard with Rossant and Fu (2023). However, with the present state of knowledge, these warnings should not be restricted to so-called 'integrated models'. Any embryoid construct that must be suspected of possessing an active potential for realizing its totipotency (under appropriate conditions) must be treated legally like a traditional embryo. It thus appears appropriate to use the term 'embryo' for embryo-like constructs made from stem cells (as proposed by Rivron *et al.* (2023)); it does not appear appropriate, however, to introduce a new legal norm that differs from the biological definition and is not covered by biological facts, just in order to ease experimental investigations.

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