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Blastocyst-like cysts sprouting autonomously from stem cell cultures: Self-organization and implantation potential

A comment on Kime et al.: Stem Cell Reports 13:485-498 (2019)

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Blastocyst-like cysts have been described to form and to bud off spontaneously *in vitro* in cultures of pluripotent/omnipotent/totipotent stem cells (PPSCs) under defined conditions using a newly designed two-phase culturing protocol (Kime et al. 2018; Kime et al. 2019). Remarkably, these so-called **„induced blastocyst-like cysts (iBLCs)“** showed self-organization and a potential for implantation in the uterus, in the used mouse model. An important technical detail seems to be to induce, via appropriate changes of culturing media supplements, a process of transition between a naïve state and a primed state of the stem cells. The described special regimen apparently introduces a critical sequence, a temporal order, into the formation of the first few differentiated vs. undifferentiated cell types, and it initiates a peculiar process of budding-off of groups of cells from the attached cell lawn, followed by self-organization within these cell groups which then start to form cysts. These blastocyst-like cysts are composed of trophoblast-like and primitive endoderm-like cells, and they seem to also preserve a niche for a **„2C-like“** type of cells within them.

That stem cell-derived blastocyst-like structures („blastoids“) can be formed by stem cells *in vitro*, or embryoids resembling germ layer or gastrulation stages („gastruloids“), or even later stage embryonic body parts, has been reported in recent years by various groups using procedures that mostly involved more or less complicated manipulations, e.g. intricate combinations of different stem cell types, i.e. a maneuver that may be called „embryo engineering“ (Harrison et al. 2017; Turner et al. 2017; Beccari et al. 2018; Rivron et al. 2018; Sozen et al. 2018; Li et al. 2019; Sozen et al. 2019; Zhang et al. 2019; Junyent et al. 2020; Veenvliet et al. 2020; Zheng et al. 2020); for a review see (White and Plachta 2020), for a discussion of the signaling and gene activation processes involved see (Posfai et al. 2020). In mammalian embryos, morphological signs for any preformation of embryonic axes are either missing or very discrete (as discussed for the mouse by (Gardner 1997; Gardner 1999)). It is still a matter of dispute whether the minute deviations from spherical or radial symmetry (which are indeed observed, like the tilt/obliquity of the inner cell mass in the mouse blastocyst, and asymmetries in the trophoblast and primitive endoderm as seen in other species) do play a role in the determination of axes (discussed in (Denker 2016)). Therefore, it is usually assumed that a process of „symmetry breaking“ is crucial for an ordered development of germ layers and of a basic body plan with its body axes. Although symmetry breaking has been observed to occur autonomously in mouse stem cell aggregates in suspension culture (van den Brink et al. 2014), it was found to be helpful in such experiments to use a degree of „engineering“ by providing some type of spatial information, e.g. to combine the various types of cells in an appropriate polar arrangement, or to apply physical constraints or specially engineered matrices. This appeared to be an important point for allowing morphogenesis to reach frequently stages that largely resemble regular embryos and their extraembryonic membranes (Harrison et al. 2017). A remarkable detail in the recent publications by Kime et al. (Kime et al. 2018; Kime et al. 2019) is, therefore, that the formation of the blastocyst-like structures did not require specific physical manipulations but occured spontaneously by a budding-like process, under their conditions, and that considerable numbers of iBLCs were produced in each culture vessel (see below).

In their detailed analysis of the composing cell types, the authors emphasize the presence of three types of cells, autonomously taking typical positions in the iBLCs, „2C-like“ cells, trophoblast-like and primitive endoderm-like cells.

„2C-like cells“ (or „extended/expanded potential stem cells“) are an interesting sub-type/sub-types of PPSCs that just recently started to receive attention in a number of labs (Ishiuchi et al. 2015; Kolodziejczyk et al. 2015; Hendrickson et al. 2017; Rodriguez-Terrones et al. 2017; Yang et al. 2017; Baker and Pera 2018; Rodriguez-Terrones et al. 2019; Genet and Torres-Padilla 2020; Hu et al. 2020; Posfai et al. 2020; Tomoda et al. 2020; Wu et al. 2020; Yang et al. 2020). In 2015, at the time when investigations on 2C-like cells just got momentum, I commented at my website (Denker 2015) with regard to one of the first publications from the Torres-Padilla group on this peculiar subspecies of PPSC (Ishiuchi et al. 2015):

„In conclusion, notwithstanding terminological considerations, the recent report by Ishiuchi et al. ... could indeed provide a stimulus to investigate experimentally whether and under what circumstances induced 2C-like cells might be able to initiate a process of early embryonic pattern formation, comparable to early blastomeres. Or would their characteristic biological property have to be addressed better not as totipotency (in the strict sense) but rather as a maximal ability to respond to external signals channelling differentiation, i.e. plasticity? It would be highly desirable to address these questions in the future by appropriate experiments, in the mouse and in nonhuman primate models.“

The recent reports by Kime et al. (Kime et al. 2018; Kime et al. 2019) are of special interest in this context. These authors employ a methodology which they had previously developed and which allows to convert primed state mouse PPSCs into the naïve state and v.v. with appropriate media supplements (Kime et al. 2016). They started their cultures with a primed-to naïve state conversion (monitored using an X chromosome reactivation marker) of mouse epiblast stem cells (EpiSCs). In such cultures, they observed the spontaneous formation of hemispherical cysts (**blastocyst-like hemispheres**) with certain features of blastocysts, i.e. a degree of blastocyst-like organization (although these half-cysts remained attached) and of lineage markers for trophoblast, embryoblast and primitive endoderm cells.

In a second, more elaborated version of the culturing protocol, they applied a two-phase regimen, with a first phase including in the medium the SMAD2/3 signaling ALK5 inhibitor SB431542 (which inhibits primed state ActivinA/TGFß signaling); in a second phase, this small molecular inhibitor is omitted, but LIF and OMPT (a synthetic lysophosphatidic acid analogue which had previously been found to enhance embryogenesis in blastocysts by activating YAP (Kime et al. 2016)) are added. With this refined two-phase culturing system they now observed the formation of sprouting aggregates of few (8-16) cells (called „iBLC precursors, iPSC-PCs“) which detached and formed free-floating blastocyst-like cysts („**induced blastocyst-like cysts, iBLCs**“). The numbers of iBLCs budding per vessel were quite variable: at least 2-5 iBLCs per well of a 6-well plate, but often more than 30 (Table S1 in (Kime et al. 2019)). These iBLCs thus formed spontaneously in their cultures, just dependent on the media change, in a typical time course. During continuing cultivation, iBLCs self-organized to a remarkable extent and differentiated trophoblast as well as primitive endoderm-like cells (with the appropriate marker expression). Importantly, these iBLCs also possessed undifferentiated cells with 2C-like cell characteristics (for a commentary focussing on the molecular aspects see (Pour and Nachman 2019)) .

A striking observation was that such iBLCs initiated early parts of an **implantation** cascade after transfer to a uterus in this mouse model, i.e. they elicited an implantation-like response (decidualization) in the endometrium and formed extraembryonic tissues. This was comparable to the initial phase of implantation events as seen with normal blastocysts transferred as a control. However, the extraembryonic membranes formed by iBLCs were grossly underdeveloped and disorganized, and the anlage of the embryo proper did not

proceed on to differentiate a basic body plan. Instead, the whole artificial „conceptuses“ became resorbed later on. In addition to blastocysts, other controls were similarly transferred to a uterus: mouse EpiSC clusters, and embryoid bodies. These latter two controls did not implant nor elicit a decidual reaction thus proving that the iBLCs had shown a remarkable blastocyst-like behaviour, i.e. a degree of specifity of their interaction with the receptive endometrium. This was obviously due to the fact that the iBLCs possessed trophoblast, in contrast to EpiSC clusters and embryoid bodies.

Kime et al. went on comparing the gene expressions of their sprouting iBLCs with those of normal blastocysts and found a number of differences in details of intensity of expression of various marker genes as well as in morphology, in spite of the fact that main marker genes of embryoblast, trophoblast and primitive endoderm were indeed expressed. So for example they describe a structural abnormality, i.e. GATA4-enriched cells bulging away. The blastocyst-like (BC-like) hemispheres mentioned in the initial part of the study were found more similar to blastocysts with respect to marker expression than the iPSCs. The authors conclude that pluripotent stem cells *„can be reprogrammed to BC-like hemispheres with striking early embryonic implications, and ... anticipate that cell conversions in such a context may be used to study early embryonic development in vitro“.* This conclusion of course appears somewhat strained, at least as long as transcriptome analyses are missing. The iBLCs, although morphologically reminding of blastocysts and also showing many molecular similarities, appeared *„imperfect and perhaps less neatly regulated than the BC-like hemispheres (e.g. PrE regulation, Xi reactivation, pluripotency)“* (Kime et al. 2019).

In my 2016 review on self-organization of stem cell colonies and of early mammalian embryos (Denker 2016) I had proposed experiments that should be of interest when asking about the potentialities of 2C-like stem cells (2CLCs) as compared to the potential of blastomeres of early embryos. Such experiments, I expected, could be of help in the ongoing discussions about the appropriate use of terms like pluripotency, totipotency and omnipotency. Commenting on the mentioned publication by (Ishiuchi et al. 2015), and referring specifically to primitive streak (PS) and basic body plan (BBP) development by clusters of PPSCs, I wrote (Denker 2016):

„The authors reported that the 2CLC stem cells resembled blastomeres isolated from two-cell stage embryos not only with regard to gene expression patterns, but also to the capacity to reactivate transcription of endogenous retroviruses, as well as to the embryo-forming capacity gained during reprogramming by nuclear transfer to oocyte cytoplasm. Both blastomeres of two-cell stage embryos are known to be totipotent in the strict sense, i.e., capable of performing complete development in vivo.“ [Today, this latter statement has to be modified, see below.] *„Thus, the question arises whether these 2CLC stem cells might express self-organization capabilities that exceed those known from traditional ESC colonies, if tested in appropriate experimental settings (e.g., in an empty zona ...). 2CLCs are much smaller than two-cell blastomeres, and they cannot be expected to possess any axis-preinformation provided via cytoplasmic determinants derived from the zygote (as postulated by one of the existing theories...). As we have discussed, self-organized early embryonic pattern formation can apparently be stimulated in colonies of stem cells by surrogate signals, e.g., asymmetries in cell densities or physical constraints, as well as the structure of the extracellular matrix. If the potential of 2CLCs would be tested by, e.g., transferring the cells into an empty zona (providing a neutral environment excluding preimplantation asymmetry signals), it could be seen whether autonomous morphogenesis would be possible or impossible in this case. Effects of the addition of an artificial local source of morphogen could be investigated. Such experiments could shed light on the question what exactly the difference might be between a (totipotent) morula and a cluster of PPSCs, in particular of 2CLCs, which is still a conundrum at this moment. Taking as an example a somewhat later stage, the same can be said about the nature of differences between a blastocyst and an engineered trophoblastic vesicle with an inside cluster of PPSCs, e.g., 2CLCs (experiments that are a variant of the chimera formation and tetraploid complementation assays). In order*

to determine how close the biological properties of 2CLCs may come to those of two-cell blastomeres, it could appear interesting to study in such experiments whether or not a regular PS (and, thus, an incipient BBP) can be formed autonomously in vitro. However, such experiments should not be performed in the human but with non-human primate PPSCs, in order to avoid any formation of a human BBP in vitro (Denker 2014; Pera et al. 2015)". [A publication that should be cited here in addition these days would be (Aach et al. 2017) because those authors address the same ethical problem.]

Now, in 2020, my remarks on the biological mechanisms involved need to be updated in several regards. First of all, recent research has shed new light on the potentiality of blastomeres in the 2-cell stage of development, and must prompt us to reconsider a role of ooplasmic constituents in normal development. These data from the Boiani lab (Casser et al. 2019) document that already the two first blastomeres of mouse embryos are not totally equipotent in most cases, contrary to previous assumptions, but rather differ in their epiblast-forming potential. Such findings must be seen in the context of theories on a developmental role of morphogens (or their precursor mRNAs) derived from the oocyte cytoplasm, which are asymmetrically localized there, so that these morphogens become segregated unequally to the blastomeres during cleavage. Theories which postulated segregation (Seidel 1952; Dalcq 1954; Seidel 1960) had been largely abandoned, however, during the last 50 years but now deserve to be reconsidered (Denker 2020). On the other hand, in contrast to early blastomeres, stem cells, including the „2C-like" variety, must be expected to have lost any oocyte-derived cytoplasmic asymmetry during passaging. In addition, it appears improbable that developmentally significant asymmetries of this type can be regained through reprogramming of fibroblasts, or at primed-to-naïve (and back) conversion of stem cells in culture as done in the work of (Kime et al. 2019). Significant for what is going on in cultures of stem cells is, on the other hand, that the asymmetry signals which probably govern morphogenetic patterning (the development of embryonic axes) *in vivo*, can obviously be replaced by surrogate asymmetries in stem cell cultures (Denker 2004). Thus it is to be expected that in future experiments on the formation of blastocyst-like constructs from stem cells as in (Kime et al. 2019), ways will be found how to increase the developmental potential beyond early implantation competence, i.e. up to more normal morphologies of extraembryonic annexes, as well as to gastrulation and basic body plan formation in the anlage of the embryo proper. This may be done by adding asymmetry cues *locally* to stem cell constructs, using morphogens (as acting *in vivo*, mostly still to be identified) or surrogate chemical or physical asymmetry factors as discussed earlier (Denker 2004). „Embryo-engineering" experiments of this type may help investigating the nature and mode of action of such asymmetry signals as involved in regular development, and this will certainly be pursued in the near future. As I suggested before (see above, (Denker 2016)), providing a protective, neutral, non-adhesive surrounding for such constructs, after they have been initiated, seems to be an essential condition in such experiments (artificial zona pellucida, suspension culture, non-adhesive matrix gels). This type of experimental settings could also be used as a test for any potential for autonomous development of the constructs, i.e. to probe for independence vs. dependence on instructions from external sources. Such tests might, therefore, provide criteria for arriving at a more stringent definition of the terms: totipotency, omnipotency and pluripotency.

In conclusion, these recent studies (Casser et al. 2019; Kime et al. 2019) lead to emphasizing the following views about developing early embryonic systems, i.e. early embryos as compared with totipotent/omnipotent/pluripotent stem cell clusters, *in vivo* and *in vitro*:

-- ***In vivo* embryogenesis**: The cytoplasm of the oocyte/zygote provides, by its asymmetrical structure and distribution of relevant molecules, an important degree of preinformation that is relevant for ordered development of early embryonic axes, not only in non-mammalian species but also in mammals. This preinformation becomes segregated during cleavage and ensures the development of structural order in the blastocyst and, in

due course, during implantation up to the basic body plan stage. These early developmental processes are basically autonomous (independent on any specific informations from the outside, the uterus) meaning that the system is already complete. However, the involved processes are very sensitive to many kinds of noises so that mammalian morphogenesis needs a special, protective environment (shielding off of differentiation signaling from outside). If this shield is insufficient (e.g. at ectopic implantation sites), disordered development ensues, and a teratoma forms instead of a basic body plan.
-- ***In vitro,* stem cell aggregates**: The high self-organizing potential of PPSCs can lead to the development of various degrees of order, depending of the culturing conditions provided. Under most conditions, disordered differentiation of various cell types or of organoid fragments is observed, comparable to a teratoma. However, a high degree of order, i.e. blastocyst-like or gastrulation stage-like, and even basic body plan-like stages, can be reached under certain conditions, in particular if naïve type / 2C-like PPSC are involved, *in vitro*. Since implantation initiation is dependent on trophoblast (not embryoblast)-derived signalling (and endometrial receptivity), trophoblast differentiating stem cell models can even initiate early parts of an implantation cascade. Development of a normal basic body plan is in most cases deficient in stem cell clusters, because of lack of the guidance by ordered asymmetry cues as provided in normal embryos from earliest stages on by segregation of ooplasmic constituents. However, PPSCs respond sensitively to environmental signals including cell-cell (neighbouring cells in the cluster) and cell-matrix (culture vessel geometry) interactions. Details, like the number of cells involved, and the time course of initiated differentiation events, are also important. Approaches which take these peculiarities into consideration can lead to improved embryo-like constructs, specifically when artificial asymmetry cues are appropriately applied. A great variety of more or less embryo-like constructs can thus be expected to be able to form *in vitro* depending on details of the conditions chosen, which is indeed already widely documented in the literature. It is to be expected that finally full viability of such constructs can be reached.

Ethical implications: If an *in vitro* system allows for the production of large numbers of stem cell-derived embryoid constructs, as in the experiments described by (Kime et al. 2019), this type of research is much facilicated. Such experiments should, however, not be done with human stem cells, for ethical reasons, since what is happening here in stem cell colonies, are self-organization processes which are the biological basis of individuation. In their commentary, Pour and Nachman (Pour and Nachman 2019) recommend to take advantage of the methodology developed by (Kime et al. 2019) and to produce large numbers of *human* iBLCs from induced PPSCs (iPS cells) in infertility clinics: for diagnostic purposes, and possibly for the personalization of therapies, e.g. by studying the properties of iBLCs formed *„from iPS cells derived from individuals with repeated failures of early pregnancy“.* However, researchers and clinicians should rather be warned not to follow this path leading towards instrumentalization of early human embryonic constructs, since these embryoids are getting closer and closer to developing autonomy and viability. I urge to critically reconsider the ethical implications of producing and of using such human embryoid constructs.

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