DENKER, H.-W.:

Recent embryo twinning data prompt to reconsider theories on a crucial role of segregation of oocyte cytoplasmic areals in mammals

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Molecular Human Reproduction https://academic.oup.com/molehr/advance-article/doi/10.1093/molehr/gaz051/5559458)

A recent publication from the Boiani laboratory (Casser et al. 2019) can be expected to trigger a re-thinking about existing concepts on mechanisms of early mammalian development, and will probably kick off a wave of interesting follow-up studies. In brief, the development of sister 2-cell stage blastomeres (when taken apart from each other) is not easily compatible with those theories about early mammalian development which are most widely accepted at the present time (inside-outside hypothesis, polarization hypothesis).

Previous findings of these authors

The data these authors are now presenting extend previous work from the same group (Casser et al. 2017; Casser et al. 2018) in which they had reported the results of 2-cell stage splitting experiments performed in the mouse, and had subsequently discussed these in the context of a meta-analysis of literature data on gene expression in 2-cell stages. Their own experiments had shown that, contrary to expectation, the potential of the first two blastomeres for autonomous development, if tested after isolation, is not equal, and that this inequality manifests itself in a difference in the number of epiblast (EPI) cells produced by the resulting blastocysts later on: In the majority of cases (73%) only one member of the pair of split embryos differentiated a sufficient number of EPI cells while the other member did not. Only in a minority of cases (23%) did both members of the pair produce a normal population of EPI cells (concordant vs. discordant pairs (Biase et al. 2014)). The number of EPI cells is known to be critical for full developmental competence, i.e. the execution of a basic body plan (Yan et al. 2003). It was thus concluded that in most of the cases one of the two separated 2-cell stage blastomeres was deficient and did not have full developmental competence (addressed as **totipotency**). This observation was interpreted as indicating unequal segregation of potentiality by the first cleavage division. The results of the experimentation were found to be constant under variable experimental conditions, and were insensitive to parameters of oocyte quality as well as culture environment (various culture media). Only one of the conclusions these authors were drawing did not appear convincing: They felt that they could exclude inheritance of the sperm entry point (SEP) by one of the two blastomeres to be a reason for the observed differences. The authors tried to test for this by doing comparable splitting experiments with cloned embryos produced by somatic cell nuclear transfer (SCNT) instead of normal fertilization by sperm, and they observed the same type of results in spite of the fact that these embryos had not experienced sperm penetration. Caution was in place, however, with such a conclusion, because the modification of oocyte cytoplasmic architecture at zygote formation (occurring at normal fertilization due to a calcium wave originating from the point of sperm

entrance and leading to cytoplasmic reorganization, reviewed in (Denker 2004)), can be elicited somewhat similarly, so to say as a surrogate, by other local membrane alterations. It can thus also be expected to occur in a comparable way by introducing another nucleus through the oocyte membrane when performing SCNT.

The new data

The new study (Casser et al. 2019), again in the mouse model, not only confirms the main previous findings but adds to them an important molecular aspect (see below). Most interestingly with regard to theories of development, the conclusions to be derived from these data, together with those from the previous investigation (Casser et al. 2017), are quite in contrast to the widespread believe that the blastomeres of the two-cell stage (or possibly up to the 8-cell stage) have equal developmental potential (totipotency), and that they receive the necessary signals for differentiation and pattern formation only later on by cell-cell interactions. The new findings are indeed not easily compatible with those theories about early mammalian development which are most widely accepted at the present time (inside-outside hypothesis, polarization hypothesis). I will address this latter point in somewhat more detail further below.

The main new results of this study (Casser et al. 2019) are as follows:

- The authors have now added a series of splitting experiments with parthenotes, lacking an SEP as well as any asymmetry introduced by SCNT. Interestingly, differences in potentiality between the first two blastomeres with regard to EPI formation (and subsequent basic body plan development, i.e. totipotency) were found also in this case, like in embryos derived from normally fertilized eggs. They can, therefore, only be explained by differential inheritance of a (probably cytoplasmic) asymmetry already present in the oocyte, by the individual blastomeres.
- A molecular substrate for this imbalance between blastomeres is now identified as the subcellular distribution of a gene product, the EPI-related gene *Cops3* (Yan et al. 2003). This is shown using mRNA FISH in super-resolution mode confocal microscopy. Since the unequal distribution of this mRNA was found to be alpha-amanitin-resistant, this imbalance does not result from de-novo transcription after the start of embryonic genome activation (EGA) but from processes taking place before fertilization (or arteficial egg activation/parthenogenesis), i.e. during oogenesis. An asymmetrical distribution of *Cops3* is indeed not only found in the 2-cell stage but also already in the oocyte. The functional role that *Cops3* may play already in this early developmental phase still remains to be clarified, but at least it serves as a very interesting early molecular marker. It will be fascinating to see how many other genes besides *Cops3* may also exhibit an asymmetrical distribution of transcript in the oocyte or blastomeres.

The authors conclude from their observations that their results „point to aspects of cytoplasmic organization of the mouse oocyte that segregate unequally to blastomeres during cleavage“. Since the first cleavage plane is known to occur in different angles in different embryos, it is indeed to be expected that areas of oocyte cytoplasm will end up in the two first blastomeres in varying proportions, i.e. distributed equally or unequally. And if these oocyte areals do harbour differing quantities of

constituents that are developmentally relevant, blastomeres should not have equal potentiality in all cases.

In discussing these results, the authors consider *Cops3* an example for unequally distributed mRNAs (or other potential cytoplasmic asymmetries) which will certainly be searched for in more detail in follow-up experiments. *Cops3* was selected from a list of genes that are part of the regulatory network of the EPI (also including *Essb, Foxd3, Gbx2, Klf2, Klf4, Nanog, Pou5f1/Oct4, Sall4, Sox2 Tdgf1/Cripto and Tfcp2/1). Cops3* stands out and deserves prime interest at present since its uneven distribution is, remarkably, not comparably shared by the other tested gene products, *Oct4* and *Gapdh.* So far only few other studies have clearly and consistently shown differences between the first two blastomeres on the molecular level, in mammalian embryos (for a review including later cleavage stages, see (Ajduk and Zernicka-Goetz 2016)). Two interesting examples for 2-cell stage asymmetries are *LincGET* and *Neat1,* long non-coding RNAs. *LincGET* was found differentially expressed in the two nuclei of the 2-cell stage. Overexpression of *LincGET* biases cells to an embryoblast fate (Wang et al. 2018). Another gene of interest is *Neat1* (required for *CARM1* association with nuclear paraspeckles which are asymmetrically distributed in the 2-cell stage; depletion of *Neat1* results in promotion of a trophoblast lineage bias as shown by *Cdx2* expression) (Hupalowska et al. 2018)). These latter two observations document differences seen in properties of 2-cell nuclei, not cytoplasm, however. They could of course result from inheritance of different parts of oocyte cytoplasm with unequal signaling properties; whether such a cascade is at work here would still have to be investigated, however. For the time being, *Cops3* appears to be the only molecular marker for a developmentally significant gene product that points directly to segregation of differential portions of oocyte cytoplasm to the first two blastomeres, but others may be found in subsequent investigations (There is evidence for segregation at later cleavage stages, concerning the inheritance of animal vs. vegetal cytoplasm of the oocyte; for a review see (Ajduk and Zernicka-Goetz 2016)). The extent to which each of the two first blastomeres receives this mRNA obviously differs according to the positioning of the first cleavage plane, as does the developmental potential (formation of EPI and its derivatives, and the subsequent formation of a basic body plan). The unequal distributions in the various parts of oocyte cytoplasm may of course depend on and reflect the distribution of RNA binding proteins which will most probably be searched for in the future.

Relevance for theories on development

These new data and their implications must be seen in the context of ideas about mechanisms of differentiation and the formation of axes and germ layers in early mammalian development, as have prevailed during the last about 50 years. These theories have ethical implications, not only for theories on twinning but also for implications of blastomere biopsy (since in some countries, like Germany, the isolation and handling of totipotent human blastomeres would be illegal, even for diagnostic purposes). Specifically, we should see reason to reconsider the segregation hypothesis vs. the inside-outside/polarization hypothesis (reviewed in (Denker 1976; Denker 1981; Denker 1983); for a recent model combining the mechanisms see (Chen et al. 2018)).

Theories previously discussed for mechanisms governing early mammalian development were, roughly speaking, of two types, differing in the involved mechanisms they are focussing on:
(1) **Segregation:** Instructory factors/molecules are distributed unequally (asymmetrically) in the cytoplasm of the oocyte/zygote; they are governing (directly or indirectly) the determination of the divergent cell types (trophoblast, embryoblast) as well as primary axes (embryonic-abembryonic = em-abem; perhaps also anterior-posterior = a-p?). The individual blastomeres receive unequal quantities of these factors/molecules during cleavage. Their action within these individual blastomeres is later on followed by cell-cell interactions leading to elaboration of fine-tuned germ layer and body plan formation. The (molecular) nature of such factors remained so far unclear.
(2) **Cell-cell interaction only:** Cell type and axis determination are independent of oocyte/zygote architecture, and of segregation of factors/molecules; determination of cell types and formation of body axes depend exclusively on later occurring cell-cell interactions (**inside-outside hypothesis**, **polarization hypothesis**) (discussed in Denker 1976; Denker 1983) . The formation of the embryonic vs. the abembryonic pole (em-abem axis) is thought in these concepts to occur at a non-predetermined location: The positioning of the inner cell mass (ICM, embryonic knot) is imagined to occur fortuitously, when the blastocyst cavity starts to expand, and the different behaviour and fate of polar trophoblast (Rauber's layer) vs. embryoblast is assumed to result from subsequent cell-cell interactions between the former and the latter group of cells (by signal molecule exchange, for which data do indeed exist at least for later stages). The determination of the a-p axis was thought by many researchers to depend on interactions with the endometrium at implantation, a concept which, however, is not substantiated by experimental findings (discussed in (Denker 2016); see also below).

Historically, the segregation theory (1) was the classical one, based on morphological, histochemical as well as experimental (deletion experiments) data ((Dalcq 1954) (Seidel 1952; Seidel 1960) (Tarkowski 1959), for additional references and a discussion see (Denker 1972; Denker 1976; Denker 1983; Denker 2016), for a recent review see (Boiani et al. 2019)). During the last about 50 years, this theory was, however, largely abandoned in favour of theories negating a developmentally relevant role of segregation, i.e. the inside-outside hypothesis (Tarkowski and Wroblewska 1967) and a variant of it, the polarization hypothesis (Johnson et al. 1981) (2). This change of opinion occurred under the impression of numerous publications demonstrating a vast regulative capacity of early mammalian embryos. A slow process of change back started in the 1990s, but it remained so far incomplete, and it concentrated initially not so much on cell type specification but rather on determination of embryonic axes. This was first discussed for the anterior-posterior (a-p) axis (Gardner et al. 1992). At that timepoint it still appeared uncertain (at least for those authors that were focussing on the mouse model) whether a role for egg organization could be considered, or whether a-p axis determination would depend entirely on signals received at and by blastocyst implantation. The latter possibility was, for many years, taken for granted by most investigators, and it was only later that findings accumulated which demonstrated autonomy of a-p axis development as seen in unattached blastocysts in vitro. The postulate of such a morphogenetic role for the uterus must now be considered obsolete (discussed in (Denker 2016)). In the following years, in particular Richard Gardner (Gardner 1996) (and later also Magdalena Zernicka-Goetz and others (Zernicka-Goetz 2005) (Plusa et al. 2005)

(Fujimori et al. 2009)) started to focus also on the earlier occurring process, the development of the embryonic-abembryonic (em-abem) axis, i.e. the precursor of the dorsoventral axis. The existing literature of those years makes it quite obvious that re-considering a possible role of cytoplasmic axis determinants contributed by oocyte (zygote) cytoplasm, was an audacious endeavour because at that time the majority of researchers working with the mouse model was still very much under the impression of the dominating inside-outside/polarization theory. This re-thinking was once again started by Richard Gardner (Gardner 1996). In addressing the known role of egg architecture for axis development in non-mammalian species, Gardner (Gardner 1996) remarks: *„Denker (1976, 1981, 1983) is notable among those who have reviewed the evidence in repeatedly challenging the view that differentiation in early mammalian embryos is rooted entirely in events that take place after the onset of cleavage.“*

At the present time point, an agreement about the mechanisms of axis determination in the mammal has not quite been reached yet, although controversies (even battles that were fought out viciously in the literature) have somewhat calmed in recent years. The case for a role of oocyte asymmetries appears to be strong for at least the development of the em-abem axis ((Gardner and Davies 2006); for a recent review focussing on polarity and cleavage division order see (Ajduk and Zernicka-Goetz 2016)). For the a-p axis, experimental evidence for transmission of relevant asymmetries derived from the oocyte/zygote is much less strong. The classical interpretations of histochemical findings by Dalcq (Dalcq 1954) as well as the observations on the interrelationships of cleavage order, cell fate and asymmetries of the blastocyst (Zernicka-Goetz 2006) suggest that there might be a connection between spatial informations with regard to both, the em-abem and the a-p axis. At least it has become rather clear that formation of the a-p axis is not dependent on implantation in the uterus but is embryo-autonomous (for literature see (Denker 2016)). The typical morphological asymmetry of the mouse blastocyst („tilt“, and oval shape of the embryoblast, related to aspects of oocyte bilateral symmetry, (Gardner and Davies 2006)) may indeed point to a transmission of a-p axis-relevant spatial information from the oocyte/zygote onto later developmental stages. It is unknown at present, however, whether and how this structural information may be encoded by asymmetries on the molecular level. The genes and signalling processes involved in cell polarity development and in the cell-cell interactions which mediate this information transfer and evolution of complexity, are under scrutiny at present (Ajduk and Zernicka-Goetz 2016; Chen et al. 2018). These investigations focus on trophoblast vs. embryoblast and em-abem axis formation. A question that also awaits answering in this context is that of possible differences between mammalian species, i.e. whether any such molecular pre-patterns and the signalling cascades they may initiate could be the same or different, in species like the mouse with germ layer inversion (egg cylinder, „symmetry breaking“ to reach the definitive positioning of the anterior visceral endoderm, AVE) as compared to species with a flat embryonic disc like the rabbit and the human (Idkowiak et al. 2004; Rossant and Tam 2009; Takaoka and Hamada 2012).

The authors of the present paper (Casser et al. 2019) underline that, while their results are clearly in favour of the segregation theory and not the inside-outside/polarization theory, these observations do

not argue against the known impressive regulative capacities of the early mammalian embryo. This view comes very close to the previous proposal (Denker 1976; Denker 1981; Denker 1983) that a combination of both mechanistic principles, segregation of morphogenetic factors and cell-cell interactions, is at work here. Recently a model has been proposed on how stochastic and deterministic principles may be acting in combination in early mammalian development (Chen et al. 2018). In the same way referring to the Turing/Meinhardt model, it had already been discussed before how axis development may be initiated by surrogate asymmetries occurring *in vitro* in colonies of stem cells (Denker 2004). The present publication (Casser et al. 2019) does not only add an interesting molecular dimension to all these discussions (asymmetrical distribution and segregation of a maternal mRNA, *Cops3,* correlated with findings on potentiality), but should serve as an opportunity to reconsider the term **totipotency**. The meaning given to this term has been shifting in the past (Denker 2012). In the most comprehensive version, this term refers to the potential to form a complete, viable embryo (principally capable of developing into a healthy newborn). However, in stem cell research, where particular interest tends to focus on the cell type differentiation capacities, not on formation of a complete embryonic body with its high degree of order (axes etc.), the term totipotency is often used interchangeably with **pluripotency**. Another term, **omnipotency** (Denker 2014) (or „plenipotency“, (Condic 2014)), has been proposed for those stem cell types which can differentiate into all (not only a few) cell types, but may not (or only in rare exceptions) be able to form autonomously a complete, viable embryonic body, without any additional intervention. The rapidly increasing number of recent publications on the production of embryoid structures, coming closer and closer to deserving the term „synthetic embryos“, from stem cells ((Denker 2016); cf. also „SHEEFs“ = „Synthetic Human Entities with Embryo-like Features“, (Aach et al. 2017)) draws our attention to the role of asymmetry centers, and how they are involved in the instruction for the formation of axes during self-organization of stem cell colonies (Denker 2004). How complex the cascades of processes (that may finally lead to the organization of a basic body plan) are, what the role of cytoplasmic asymmetry inherited from the oocyte/zygote may be in normal embryonic development, how exogenic, „surrogate“ asymmetries may be able to replace this missing oocyte-derived information in stem cell-derived embryoids, and what degrees of independence from such pre-information (i.e. a degree of autonomy) could arise in groups of pluripotent/omnipotent stem cells under what conditions, all these are questions that can be attacked experimentally now with the help of the large arsenal of in vitro techniques that have become available recently (Deglincerti et al. 2016; Shahbazi et al. 2016; Harrison et al. 2017; Shao et al. 2017; Martyn et al. 2018; Sozen et al. 2018; Britton et al. 2019; Kime et al. 2019; Li et al. 2019; Sagy et al. 2019; Zheng et al. 2019) (Unfortunately, it is often overlooked these days that this type of experiments should NOT be done with human stem cells, but only in the mouse and non-human primate models, for ethical reasons).

The Casser et al. paper (Casser et al. 2019) provides information on very early segregation of a mRNA (*Cops3*), most probably being just one example of a series of other candidates for factors that are still unknown. The developmental role which this factor is playing does not become apparent yet immediately at the very early stages, i.e. at cleavage and blastocyst formation, but rather at a much later stage of development: the formation of a sufficient number of EPI cells, a prerequisite for

successful development from the germ layer stage on (and thus for elaboration of the basic body plan). In presenting these new findings, the paper moves segregation out of the realm of speculation, with regard to mammalian development, and moves it into the area of experimentally testable ideas based on molecular events. The case of *Cops3* offers a proof of principle that the cytoarchitecture of the (mouse) oocyte matters for development, more than we thought before. However, what is still not clear yet is whether *Cops3* segregation data can directly help with illuminating processes of cell type and axes determination in the earliest developmental stages, i.e. during cleavage and differentiation of trophoblast vs. embryoblast (em-abem axis development) or a-p axis formation. It is to be expected, on the other hand, that the present publication (Casser et al. 2019) will stimulate an active search for other factors possibly also undergoing segregation, and their possible role in axis formation processes. In any case, this paper gives reason to contemplate, once again, the various definitions for totipotency.

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