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PERSPECTIVE

In preprints: opportunities to unravel the earliest stages of human development using stem cell-based embryo models

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Several exciting advances have enabled the derivation of stem cell-based embryo-like models (SCBEMs). Such models allow us to interrogate previously intractable questions in developmental biology and ask hypothesis-driven fundamental questions such as how the body plan forms, how tissue types interact and how transcriptomic, proteomic and metabolic states influence early human development. SCBEMs provide researchers with scalable, accessible and experimentally tractable systems when access to human embryos is limited or insufficient. SCBEM is an umbrella term, describing a diverse range of models produced through different protocols from self-organised stem cells. Crucially, none of the current models are 'equivalent' to human embryos; different SCBEM models represent specific aspects of development and to varying degrees. For example, SCBEMs may represent some, but not all, of the components of the early conceptus, whereas others model specific stages of development. Other SCBEMs might have the right cell types but in a disorganised, disproportionate or morphologically dysplastic state. Like any 'toolbox', human SCBEMs are powerful precisely because they represent a variety of 'tools' to ask a range of questions.

Recently, several preprints have described new human SCBEMs, specifically at the postimplantation stage. This is a notable step forward, but builds on an established foundation of previous work, because it extends the time window modelled by preimplantation-stage blastoids (Kagawa et al., 2022), which recreate blastocyst structure and *in vitro* uterine tissue interactions, but do not progress through gastrulation (Santis et al., 2023 preprint). Likewise, several postimplantation human SCBEMs have already been described including 2D micropatterns (Warmflash et al., 2014), postimplantation amniotic sac embryoids (Zheng et al., 2019) and 3D gastruloids (Moris et al., 2020), which model the symmetry-breaking events of gastrulation and body-plan organisation. But such models are not formed with extra-embryonic tissues, such as the trophectoderm or hypoblast, and so do not represent the full embryo with extra-embryonic components. Therefore, the work reported in recent preprints offers important logical steps toward human SCBEMs that are more integrated (they have, to varying degrees, both embryonic and extra-embryonic components) and that may mirror stages further along the developmental timeline than current integrated models offer. We briefly describe and compare these preprinted models below, without attempting to provide comprehensive peer review, but to summarise and comment on what is reported in relation to the rest of the field.

Weatherbee and colleagues (2023) described a so-called 'human embryoid', in which the forced overexpression of transgenes induced naïve human pluripotent stem cells (hPSCs) to differentiate towards three different tissue types: the pluripotent epiblast (no transgene induction), the hypoblast (with inducible *GATA6* and *SOX17*) and what they reasoned would represent the trophectoderm [with inducible *GATA3* and *AP2g* (also known as *TFAP2C*)]. Induced cells were mixed in a ratio of 1:1:2, respectively, and aggregated, yielding 3D structures under conditions that promoted their differentiation. What resulted was a lumenised epiblast-like domain surrounded by a single-cell layered GATA3/AP2g domain, with an intermediate population of hypoblast-like cells (Fig. 1A). Of note, the induced GATA3/AP2g cells did not exhibit a trophectoderm-like signature, as assessed by single-cell transcriptomic analysis, implying that these cells provided a supporting role in the embryoid, but did not serve as trophoblast-like cells, nor did the cells persist in the developing structures. However, the structures did show evidence of cell types including an emergent amnion population, early brachyury-expressing (TBXT+) mesoderm (which is often associated with primitive streak-stage embryos) and extra-embryonic mesenchyme, as well as hypoblast and primordial germ cell-like cells (PGCLCs). It is remarkable that these structures can generate so many cell types that mirror those observed in an early postimplantation embryo, given the relatively simple arrangement of tissues in this model. However, it also provides important cautionary advice against relying on a handful of 'marker genes' to identify cell types or tissues of interest as, particularly in an *in vitro* context, these do not always align to the full transcriptomic signature of embryonic equivalents.

Hislop and colleagues (2023 preprint) were similarly interested in the relationship between the epiblast and hypoblast lineages. They used 2D cultures to generate so-called 'iDiscoids'. These structures were derived from induced pluripotent stem cells with an inducible *GATA6* transgene, co-cultured with wild-type hPSCs, before activation of the transgene. Subsequently, wild-type hPSCs form small lumenised discs overlaid by transgene-induced cells (Fig. 1B). The lumenised cavity contained a squamous amnion-like population in contact with the dish substrate, and a columnar epiblast that is adjacent to the hypoblast-like overlaid cells. With sustained culture, these structures generated a TBXT+ domain and extra-embryonic mesodermal cells. One of the surprises of this work was the observation that the anterior hypoblast (also called the anterior visceral endoderm or AVE) was polarised in 42% of structures, but that this could occur equally on the opposing side to TBXT+ epiblast cells or the same side. This seems to contradict observations in embryos of several other species, including mouse, where the opposing orientation of the AVE and primitive streak domain is key to early symmetry-breaking and anteroposterior axis formation. Notably, the 'iDiscoid' SCBEMs exhibited some evidence of haematopoietic cell generation, associated with the

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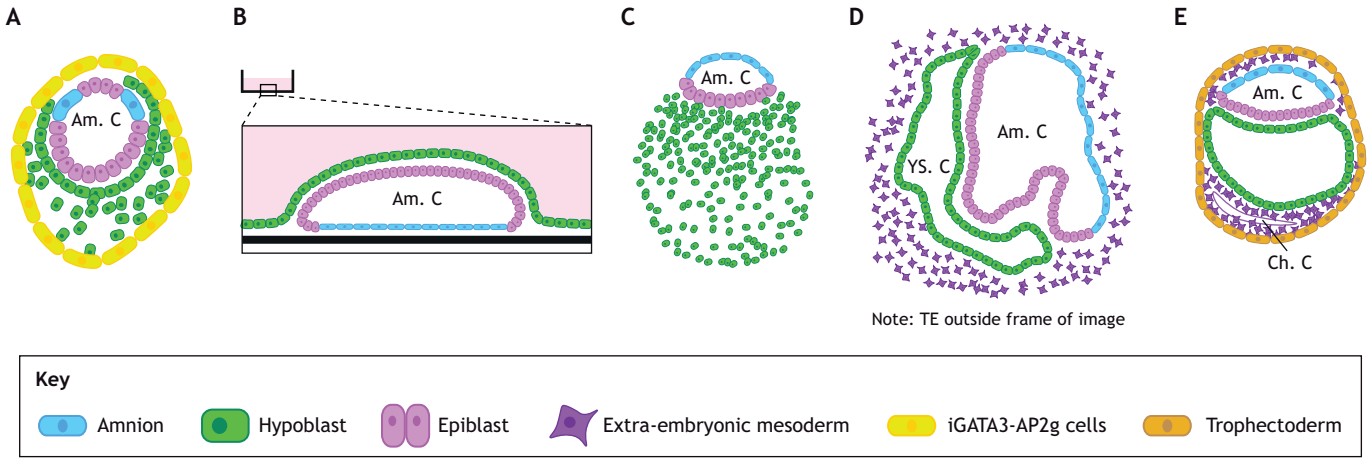

Fig. 1. Schematic representation of human stem cell-based embryo models (SCBEMs) preprinted recently, showing one example of the structural architecture of each system. To see the full representation of each model, we refer the reader back to the original preprints. (A) 'Human embryoid model' adapted from figure 2G from Weatherbee et al. (2023). (B) 'iDiscoid' adapted from figure 2C from Hislop et al. (2023 preprint). (C) 'Non-integrated human pluripotent stem cell-based gastruloid model' adapted from figure 3F from Yuan et al. (2023 preprint). (D) 'Embryo-like assembloids' adapted from figure 6G from Ai et al. (2023). (E) 'Integrated synthetic embryo models' adapted from figure 4G from Oldak et al. (2023 preprint). Am. C, amnion-like cavity; Ch. C, chorionic cavity; TE, trophectoderm; YS. C, yolk sac-like cavity.

yolk sac in early mammalian embryos, indicating the tantalising prospect that SCBEMs could be used to generate rare and otherwise difficult-to-access populations of cells.

Continuing the theme of coordinated juxtaposition between the epiblast and hypoblast domains, Yuan and colleagues (2023 preprint) reported the appearance of a lumenised amniotic cavity sitting on top of a hypoblast-like domain (Fig. 1C) when hPSCs are cultured under particular culture media conditions. Like the previous two preprints, the structures generated cell types including amnion, PGCLCs and mesodermal progenitors, as well as hypoblast derivatives, in the absence of any trophectoderm cell types. Beyond this, Yuan and colleagues demonstrated a potential utility of such models by exploring the effect of exposure to Thalidomide, similar to studies in mammalian gastruloids (Mantziou et al., 2021), which impacted the formation of some tissue types and reduced overall size. Further experiments are required to prove whether this is evidence of teratogenic effects or cytotoxicity, but the overall approach highlights the potential use of SCBEMs in downstream applications including drug screening.

Ai and colleagues (2023) used cells in a naïve condition they developed, which they call AIC-N, alongside either human trophoblast stem cells or BMP-treated cells, to try to replicate the signalling network provided by extra-embryonic cells to the developing embryo-proper. By tweaking the signalling composition of the culture conditions, they could persuade the assembled cell structures towards producing their own extra-embryonic endoderm-like (XEN-like) cells, alongside several examples of amnion-like and mesoderm-like cells, as well as cells that resemble extra-embryonic mesoderm and primordial germ cell-like cells. Many of the entities contained two lumenised cavities, seemingly correlating to amnion-like and yolk sac-like structures (Fig. 1D). The strength of this research is their extensive manipulation of the signalling landscape to prospectively direct cell fate decisions, which informs their experimental design. However, the degree to which the cell types they identify by single-cell transcriptomics correlate to cognate spatially organised tissues requires further characterisation.

Finally, Oldak and colleagues (2023 preprint) developed models where naïve hPSCs are aggregated with hypoblast-like and trophectoderm (TE)-like cells (also derived from human enhanced naïve stem cell media conditions without any genetic modification) in a 1:1:3 ratio, respectively, and allowed to self-organise. The resultant structures developed lumenised amnion-like and yolk sac-like cavities, surrounded by cyto- and syncytio-trophoblast supporting cells (Fig. 1E). The structures developed TBXT+ and PGCLC populations but also formed extra-embryonic mesenchymal cells. In addition, this system appeared to support the formation of a chorionic cavity. The strength of the work is in the remarkable organisation evident in some of the structures, which closely resembles that of the early postimplantation human embryo, although this appears to be a very rare event, with the authors noting that an organised morphology occurs in only ∼2.9% of structures on day 6.

Overall, these papers [alongside others published (e.g. Pedroza et al., 2023; Liu et al., 2023) without preprints and so are not included here] reiterate the astonishing power of hPSCs to self-organise into structures that resemble embryos when provided with the right environment and supporting cell populations. It is important to note that none of the models presented here went through a blastocyst-like stage, and instead reached a postimplantation-equivalent stage in a completely non-canonical manner. In addition, none of the models could go much beyond the earliest stages of symmetry breaking and mesoderm emergence, so they clearly mark only a snapshot in developmental time. This realisation also has important implications for our regulatory governance of such models because it is increasingly clear that SCBEMs do not progress linearly through development in the same way as embryos; therefore, regulations such as the day 14 rule (Borggrefe and Oswald, 2009) cannot be easily transferred to such structures because some stages, including fertilisation and implantation, can be 'bypassed'. Likewise, for models that possess some but not all extra-embryonic tissues, categorising them in the existing system is challenging because they fall somewhere between fully integrated embryo-like models and non-integrated models (Lovell-Badge et al., 2021). At the time of writing, there is intense activity to reflect on existing research guidelines – pioneered by the International Society of Stem Cell

Research – while assessing whether additional governance frameworks are required.

Significant research effort is necessary to further characterise these new systems in parallel, and particularly to improve their reproducibility and efficiency. It will be a high priority to move beyond development and description of ever new systems and instead start using existing exciting models thoughtfully to answer important questions of the field. It is vital that the research community showcases the purpose and utility of SCBEMs to the community and wider public. This becomes more crucial considering renewed press interest and broad discussions about regulation of such models, where we must strive to clearly explain both the benefits and, crucially, the limitations of the ever-expanding toolbox of human SCBEMs.

Note added in proof

Oldak et al., 2023 has now been published as: Oldak, B., Wildschutz, E., Bondarenko, V., Comar, M.-Y., Zhao, C., Aguilera-Castrejon, A., Tarazi, S., Viukov, S., Pham, T. X. A., Ashouokhi, S. et al. (2023). Complete human day 14 post-implantation embryo models from naïve ES cells. Nature [Epub ahead of print]. doi:10.1038/s41586-023-06604-5

Competing interests

R.S. is Chair and N.M. is a member of the Governance of Stem Cell Based Embryo Models Guideline Working Group, a UK initiative led by Cambridge Reproduction to develop recommended guidelines for research with SCBEMs.

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