© 2022. Published by The Company of Biologists Ltd | Development (2022) 149, dev201354. doi:10.1242/dev.201354

PERSPECTIVE

In preprints: towards reconstituting an ovary

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Efforts towards the *in vitro* derivation of germ cells from stem cells have seen considerable progress in the past decade. The successful generation of oocytes *in vitro*, although challenging, will bring a major advance to reproductive biology – from understanding the complex developmental mechanisms of human oogenesis, to applications in disease modelling of infertility. Current attempts to achieve germ cell and somatic gonadal differentiation *in vitro* involve two key approaches: application of exogenous signals for induced differentiation paired with gonadal soma co-culture and, more recently, overexpression of candidate regulators. Here, we highlight two preprints that progress the *in vitro* differentiation of human female gonadal cells and oogonia.

Initial attempts at this endeavour involved the directed differentiation of competent pluripotent stem cells towards primordial germ cells (PGCs) – the precursors that eventually give rise to gametes. Exogenous BMP signalling is key to this, with this protocol giving rise to bona fide mouse and human PGC-like cells (m/hPGCLCs) within 4 days (Saitou and Hayashi, 2021). Subsequent *in vitro* maturation of mPGCLCs involves optional expansion with signalling molecules and co-culture with sex-matched embryonic gonadal somatic cells (Ishikura et al., 2021; Saitou and Hayashi, 2021). Co-culture is thought to provide the necessary cellular architecture and signalling niches to support full germ cell maturation (Cooke and Moris, 2021), with these laboratory-generated sperm and oocytes capable of generating offspring (Saitou and Hayashi, 2021). Furthermore, it was recently shown that *in vitro* differentiated fetal ovarian somatic cell-like cells from mouse embryonic stem cells (mESCs) can effectively substitute for isolated gonadal somatic cells in co-culture approaches (Yoshino et al., 2021). Further maturation of hPGCLCs has also been achieved with xenobiotic mouse gonadal soma co-culture, but these cells do not progress beyond fetal oogonia and prospermatogonia stages (Hwang et al., 2020; Yamashiro et al., 2018).

Although these advances are encouraging, the protocols are protracted and inefficient. However, two recent preprints report a contrasting approach to swiftly progress the *in vitro* differentiation of human ovarian cells via the overexpression of germline-related transcription factors (TFs). Both take advantage of available transcriptomes and single-cell atlases of fetal gonadal development, in combination with known candidates and gene regulatory network analyses to identify TFs implicated in oogenesis (Kramme et al., 2022 preprint) and ovarian granulosa somatic cell development (Smela et al., 2022 preprint).

Combinatorial overexpression of TFs to achieve germline differentiation without the application of external signalling molecules has been performed previously. For instance, hPGCLCs can be more efficiently specified by overexpressing *SOX17* with either *BLIMP1* (*PRDM1*) or *GATA2*/*3*/*TFAP2C* in competent cells (Kobayashi et al., 2017; Saitou and Hayashi, 2021). Another study reported the direct differentiation of oocyte-like cells from mESCs via overexpression of a combination of oocyte-specific TFs in just a few weeks (Hamazaki et al., 2021). Although these cells did not traverse a PGC fate, and did not initiate meiosis, this work demonstrated the separation of oocyte growth from prior reprogramming events through the control of stage-specific TFs.

Now, Kramme et al. (2022 preprint) have screened 47 *in silico*-identified TFs for their role in the progression of human oocyte development, starting with competent pluripotent stem cells grown in monolayer format, deviating from the traditional approach of embryoid body aggregation. They identified three TFs (*DLX5*, *HHEX* and *FIGLA*) that when singly overexpressed achieve PGCLC differentiation more efficiently than singly expressed *SOX17*, *TFAP2C* or *BLIMP1*, albeit with a continued dependence on BMP. How these compare with the combined overexpression of *SOX17* and the core germline TFs mentioned above will be interesting to explore in future.

Unexpectedly, decreased PGCLC yield was noted when the three TFs were overexpressed in combination. Notably, two of the three TFs identified are not expressed at the bipotential PGC stage *in vivo*, with *DLX5* and *FIGLA* being upregulated only later in meiotic and oogenic fetal germ cells. *FIGLA*, in particular, was one of the group of TFs that was shown previously to specify oocyte-like cells from mESCs (Hamazaki et al., 2021). To explore these unexpected findings further, the authors showed that none of the three factors is essential for PGCLC specification, based on CRISPR knockouts. With the absence of an endogenous role in PGC specification, how then could these TFs enhance PGCLC differentiation but not further maturation? The authors uncover a hint at a solution, identifying a potential non-canonical role for these TFs in occupying PGC enhancers in forced overexpression states.

In parallel, further maturation to fetal oogonia-like cells was achieved by combinatorial overexpression of *ZNF281*, *LHX8* and *SOHLH1*, based on screening reliant on fetal germline marker *DDX4* expression, in just 4 days in monolayer culture. The authors also discovered that the addition of these factors with the three above-identified PGCLC-promoting TFs as well as three fetal germline RNA-binding proteins (DAZL, DDX4, BOLL) enhanced yields of oocyte marker-expressing ($NPM2^{+}$/$DDX4^{+}$) cells, albeit at low efficiencies against heterogeneous outcomes. The induced cells nonetheless showed hints of further maturation with broad oogonia-like transcriptomes, larger cell sizes and nest-like structures, and growth under feeder-free conditions. Intriguingly, these oogonia-like cells appear to have skipped earlier migratory and gonadal PGC stages when DNA demethylation occurs, echoing similar observations in mouse induced oocyte-like cells (Hamazaki et al., 2021), suggesting a separation of epigenetic reprogramming from oogonia maturation under TF control.

In a complementary approach, Smela et al. (2022 preprint) attempted to progress PGCLC differentiation via co-culture with

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Development (2022) 149, dev201354. doi:10.1242/dev.201354

induced human female gonadal soma. Using a similar TF screening strategy followed by validation, the authors identified the combination of *NR5A1* and *RUNX1/2* overexpression as being able to efficiently induce granulosa-like cells that express early granulosa markers ($FOXL2^{+}/CD82^{+}/EpCAM^{-}$) and harbour the ability to produce estradiol. Transcriptome analysis confirmed the global upregulation of bulk gonadal granulosa markers. Crucially, reconstitution of these induced granulosa-like cells with hPGCLCs was able to support further PGCLC maturation. Imaging and single-cell RNA-sequencing profiling of these 'ovaroids' identified persistent PGCLCs together with a small number of DAZL-positive and DDX4-positive cells and indications of the onset of X-chromosome reactivation, suggesting that asynchronous maturation into gonadal PGC and early fetal germ cell states is occurring as early as 4 days of culture. Although advancement of the somatic compartment with folliculogenesis was observed by 35 days of culture, no germ cells remained. This was in contrast to more advanced maturation of oogonia-like cells when hPGCLCs were co-cultured with mouse gonadal soma (77 days of co-culture), in which additional female gonadal somatic lineages are also present (Yamashiro et al., 2018).

Despite this exciting progress in pushing the maturation protocols towards fetal germ cell and gonadal signatures while delineating the roles of TF regulators in these processes, it is clear that the task of making human eggs and supporting ovarian cells *in vitro* is very challenging. This is particularly puzzling considering that similar attempts in mice using either co-culture or TF overexpression have yielded more advanced *in vitro* gametogenesis. Fortunately, there is a wealth of transcriptomic repositories of *in vivo* human gonadal development up until the end of the second trimester to serve as guides. Indeed, the most recent single-cell atlas has provided new insights into the diversity of subpopulations, spatial dynamics and asynchrony of both human fetal germ cell and gonadal somatic lineages (Garcia-Alonso et al., 2022; Li et al., 2017), and how these processes subtly differ from those in mice. Although both preprints discussed here relied on a strategy of lineage reporters for screening for successful germ cell maturation before transcriptomic comparisons, future work might leverage deeper and more high-throughput whole transcriptome-based screening readout strategies for unbiased similarities; these could be benchmarked against transcriptomic atlases at finer fetal germ and somatic subpopulation levels, rather than relying on a few initial markers.

In addition to recapitulating transcriptomes, other hallmarks of human oogenesis can serve as benchmarks to guide accurate *in vitro* gametogenesis. In addition to X-chromosome dynamics, which the authors observed, unique signatures, such as migratory human PGCs traversing the hindgut, gonadal human PGC expansion, erasure of imprints, histone modification dynamics, fetal oocyte attrition, fetal piRNA emergence and postnatal re-methylation, should be included as some of the early maturation milestones in these protocols (Ramakrishna et al., 2021). It is interesting to note that, although TF overexpression can result in oocyte growth from mESCs, these cells fail to enter meiosis (Hamazaki et al., 2021). This may be a consequence of bypassing some of these key steps during the induction of these cells by forced TF overexpression. Although the ultimate goal of developing human gametes *in vitro* remains some way in the distance, stepwise progress will nevertheless continue to advance our understanding of the developmental mechanisms of gonadogenesis and to make headway in modelling diseases of early germline development, such as germ cell cancers and infertility.

Competing interests

The authors declare no competing or financial interests.

Funding

R.A. acknowledges the support from the Biotechnology and Biological Sciences Research Council [BB/T013575/1]. N.B.R. acknowledges an Agency for Science, Technology and Research (A*STAR) National Science Scholarship research fellowship.

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