Editorial

Another step closer to unlocking specification of primordial germ cells

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Primordial germ cells (PGCs) are the precursors of gametes and responsible for passing genetic and epigenetic information from one generation to the next. They are specified at around the time of implantation of the developing embryo to the uterine wall and eventually migrate to the genital ridge where they differentiate and undergo meiosis in a sex-dependent manner in order to become the mature eggs and sperm (1-3). While the mouse has been a reliable model for mammalian PGC development, there are notable developmental and molecular differences compared with human. Most obviously, the mouse embryo forms as an egg cylinder, and the human as a bilaminar disk (1). Recent *in vitro* models for human PGC development established by Irie *et al.*, 2015 and Sasaki *et al.* in 2015 have led to a number of studies indicating transcriptional differences of PGC specification between mouse and human (4,5). In the mouse it is accepted that Prdm14, Blimp1 (also Prdm1), and Tfap2c form a tripartite transcriptional network for PGC specification (6). Studies in the human however, have highlighted the importance of TFAP2C, BLIMP1, and surprisingly SOX17 and EOMES in PGC development (4,5,7,8). These species differences necessitate the study of PGC specification in human rather than just extrapolation of the knowledge from mouse.

While the *in vitro* direct differentiation model from human pluripotent stem cells has proven useful in studying cell-fate-determining transcription factors during PGC development, it offers limited insight into one of the biggest unanswered questions regarding PGCs: when and where in the human embryo are the PGCs specified? Studies in mouse reveal that PGCs arise at embryonic day (E) 6.25 in the posterior epiblast (9). However, ethical and technological limitations make it impossible to answer this question directly during human embryo development. Instead, studies on species that are more closely related to human evolutionarily than mouse serve as an alternative strategy to touch on the origin of PGCs. In 2016, Sasaki *et al.* published a very surprising study suggesting that PGCs are first found in the amnion around E11 in the monkey cynomolgus macaque (10). As the beginning of a life, the fertilized egg divides to form a cluster of cells to start the first dramatic cell fate separation: the inner cell mass (ICM) that will form the embryo proper and the outer trophectoderm that will form extraembryonic tissues connecting the developing embryo to the uterine wall of the mother. Then, the ICM will soon start the second cell fate decision to form the epiblast and hypoblast (also called primitive endoderm) (11). Developmentally, the amnion is derived from the epiblast and will form a membrane that covers the developing embryo. It becomes the amniotic sac after filling with amniotic fluid to provide a protective environment for the developing embryo and eventually the fetus. This finding that monkey PGCs are specified in the amnion complicates the key question regarding the location of human PGC specification. Is an amnion origin a monkey-specific mechanism or a common mechanism for primates including human?

Human PGC-like cells (PGCLCs), the *in vitro* counterpart of *in vivo* PGCs, have been successfully made from pluripotent stem cells including human embryonic stem cells (hESCs) and induced pluripotent stem cells

(hiPSCs) (4,5,7,12). Given that little is known about the molecular nature of the amnion, it is not clear whether PGCLCs are formed through an amnion-like-intermediate during induction from hESCs or hiPSCs. It is therefore important to carefully examine the specification of PGCs from additional species. A recent study by Kobayashi *et al.* entitled "Principles of early human development and germ cell program from conserved model systems" provides insightful viewpoints into the cellular origin of PGCs (13).

Using the porcine embryo which develops as a bilaminar disk similarly to human, Kobayashi *et al.* showed that PGCs are specified in the posterior epiblast at E11 induced by WNT and BMP signaling, a cellular mechanism highly resembling mouse PGC specification (13). First, they identified porcine PGCs using well-defined PGC markers in human and monkey such as SOX17 and TFAP2C and discovered that E11 is the earliest time that PGCs are clearly specified. They then traced porcine PGC development and found conserved transcription factors and epigenome reprogramming when comparing porcine embryos to monkey and human. Thus, porcine PGCs can serve as a suitable model to study the specification of PGCs from developing epiblasts. During embryonic development, the epiblast will initiate a new wave of cell fate separation to form ectoderm and mesendoderm (the progenitor of mesoderm and endoderm) at around the primitive streak stage. To functionally dissect the time points of when developing epiblasts gain and lose competency for PGC fate, Kobayashi *et al.* established an *ex vivo* system by culturing separated epiblasts from hypoblast and trophectoderm at various embryonic stages. They then tested the competency of epiblasts to form PGCs with required cytokines in the culture media. This analysis limited competent epiblasts for PGC fate to E10/11, which represents the precursor of mesendoderm (pre-mesendoderm) cell fate. Therefore, in porcine, PGCs are most likely originated from pre-mesendoderm cells of the epiblasts. Based on these findings, Kobayashi *et al.* tested if human and monkey PGC-like cells can be induced from pre-mesendoderm cells *in vitro*. Interestingly, 12 hours of pre-mesendoderm differentiation from ESCs is sufficient to induce PGCLCs both in the human and monkey system, suggesting a possible origin of PGC fate from the pre-mesendoderm cells. Shorter pre-mesendoderm differentiation (<12 hours) is not sufficient to induce germ cell fate, while longer differentiation promotes the adoption of endoderm and/or mesoderm fate and loss of PGC fate, consistent with the finding that only the developing epiblasts at the stage of pre-mesendoderm differentiation gain competency for PGC fate.

Finally, the authors further explored the transcription factors specifying human PGC fate. They focused on SOX17, TFAP2C, and BLIMP1, all of which are involved in human/monkey/porcine PGCs and mutants of these transcription factors result in loss of competency to form PGCLCs from hESCs/hiPSCs (4,5,7,8). Kobayashi *et al.* over-expressed either single or combinatorial transcription factors during the 12 hours' period of pre-mesendoderm differentiation and tested their roles in PGCLC induction. This analysis established that SOX17 and BLIMP1 together specify human PGCLCs effectively even in the absence of BMP4, suggesting that SOX17 and/or BLIMP1 are probably the most important downstream targets of BMP signaling during the specification of PGCs.

In summary, Kobayashi *et al.* traced PGC development in porcine embryos and narrowed down the time of PGC specification to E10/11, at around the time epiblasts initiate mesendoderm differentiation, and confirmed this finding by the *ex vivo* culture system using porcine embryos and the *in vitro* culture system using human and monkey ESCs. Therefore, in human, monkey, and porcine, PGC fate is probably established from pre-mesendoderm cells during epiblast differentiation at around the time of implantation. Thus, Kobayashi *et al.* have established an evolutionarily conserved PGC specification pathway in different organisms (13).

Notably, the amnion is formed at later stage during porcine embryogenesis (after primitive streak stage) compared to that of human and monkey (before primitive streak stage) (14). Therefore, posterior epiblast cells of the pre-mesendoderm differentiation are probably the only PGC-competent cells in the porcine embryos. However, this does not exclude the possibility that PGCs are specified in the amnion of monkey embryos. As mentioned in their paper, Kobayashi *et al.* acknowledged the idea that human and monkey PGCs may be specified in both the pre-mesendoderm and the amnion, a possible dual origin (13). The *ex vivo* culture system established by Kobayashi *et al.* in this study will be a suitable strategy to test the possibility of a dual origin for PGCs in monkey. Specifically, developing monkey embryos can be separated into amnion, epiblasts, hypoblasts, and trophectoderm at different stages to test their competency for PGC fate. This can also be applied to human embryos, as human embryos can be cultured in an *in vitro* attach system for up to 14 days (15,16). Additionally, if we could develop more robust and reliable models and markers for primate amnion and extra-embryonic tissue *in*

vitro, we could interrogate these cells as an intermediate to test their competency for PGC fate induction. These analyses will provide important insights about the cellular origin of human PGCs, and will expand on the knowledge of a posterior, pre-mesendoderm epiblast origin as introduced in this study.

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