Commentary

Stem Cell INVESTIGATION

Making gametes from pluripotent stem cells: embryonic stem cells or very small embryonic-like stem cells?

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The successful differentiation of mouse embryonic stem (mES) cells into haploid spermatids was recently achieved *in vitro* and published by a group led by 3 first authors including Quan Zhou, Mei Wang and Yan Yuan from China (1). It took almost 45 years to finally reach this stage since mES cells were first reported by Evans and Kaufman (2). The haploid spermatids generated were used for intra-cytoplasmic sperm injection in mouse oocytes and resulted in viable and fertile pups. This success raises hope to treat infertile patients in future. However, it will take much more time since the scientists at present are stuck on the first step itself that involves converting human ES cells into primordial germ cells-like cells (PGCLCs). Just recently Surani's group reported that SOX17 plays a crucial role in specifying human ES cells into PGCLCs (3). It is basically difficult to convert ES cells into PGCLCs *in vitro* since it involves epigenetic reprogramming in addition to altered gene expression. Once a robust protocol is established to obtain PGCLCs from ES cells, they can easily be differentiated further into gametes. Thus it will be a while before gametes are obtained starting with human ES cells as concluded recently (4,5).

However, Zhou *et al*. (1) have achieved a major advance starting with mES cells, in obtaining spermatids which undergo meiosis *in vitro* and later produce viable and fertile pups. Their approach to achieve this success was very systematic and involved 3 major steps. First step was to differentiate transgenic mES cell lines into PGCLCs. The cells were transgenic for fluorescence reporter proteins under the control of regulatory elements of germ cell markers. This allowed them to easily track differentiation of mES cells into PGCLCs expressing STELLA *in vitro* associated with possible erasure of imprinting of parentally imprinted genes Snrpn and H19. In the second step, they cultured the PGCLCs along with neonatal testicular somatic cells in the presence of FSH/bovine pituitary extract/testosterone. Meiosis was tracked elegantly *in vitro* by studying sequential expression of specific markers. Initially chromosomal synapsis and DNA double stranded breaks and their resolution by homologous recombination repair was tracked by studying expression of SPO11 and RAD51. Expression pattern of phosphorylated H2A histone family member X recapitulated meiosis progression as it was broadly distributed throughout the nucleus on D8 reflecting an association with double stranded breaks in DNA and later focal appearance on sex chromosomes suggested completion of synapsis. The nucleus showed expression of SYCP1 and SYCP3. Later an up-regulation of meiotic markers Dmc1, Stra8 and Sycp3 was observed by D10 followed by up-regulation of transcripts specific for haploid cells including Prm1, haprin and acrosin. The *in vitro* generated spermatids were used for ICSI and basically success to obtain live pups in their IVF program was 9% in normal mice compared to 2.8% from the spermatids obtained from mES cells. These results need to be replicated by independent groups.

This low rate of pregnancy outcome using mES cells derived gametes reflects a serious scientific hurdle before this work could be translated into a clinical setting and possibly reflects inappropriate epigenetic status of the spermatids obtained in culture. A similar success to obtain spermatids from human ES cells has been reported in the past by Moore's group (6) but they could not test the derived spermatid further for ethical reasons. As mentioned above, manipulating epigenetic status of a cell in a controlled manner is difficult compared to modifying

gene expression on exposure of cells to a cocktail of growth factors and cytokines. It is intriguing that similarly the pancreatic islet progenitors derived from human ES cells have a very different epigenetic status compared to adult pancreas (7). ES/iPS cells are falling short (possibly because of their epigenetic profile) in that they tend to give rise to their fetal counterparts (8-10) and may not be really useful to regenerate adult organs. This is the underlying reason why the field of ES/iPS cells for regenerating age-related diseases has not moved as was expected.

We had earlier discussed that rather than ES cells [obtained from the inner cell mass (ICM) of blastocyst-stage embryo], very small embryonic-like stem cells (VSELs) in adult gonads, which are equivalent to PGCs (primordial germ cells obtained from epiblast-stage embryo), may be better stem cell candidates to make gametes (11). Based on their ontogeny, VSELs are relatively more mature developmentally as well as epigenetically compared to ES cells and thus spontaneously differentiate into sperm (12) and eggs (13-15) *in vitro*. Similarly we have reported that VSELs spontaneously regenerate adult mouse pancreas after partial pancreatectomy (16). Compared to ES/iPS cells which are being exploited to yield islet progenitors in culture, VSELs have the ability to regenerate both acinar cells and islets in the pancreas. More importantly, VSELs will bring about endogenous regeneration which is much more physiological compared to ES/iPS cells which are being used to generate progenitors for cell therapy.

The VSELs exist in adult ovary (13-15) and testis (17-20) in addition to the tissue specific progenitors including ovary stem cells (OSCs) in ovary and spermatogonial stem cells (SSCs) in testis. VSELs survive oncotherapy in both mice (15,18) and human (21) gonads because of their quiescent nature whereas OSCs/SSCs and germ cells get destroyed since oncotherapy basically targets actively dividing cells. They are also reported in non-functional human gonads (13,20). These surviving, endogenous VSELs in azoospermic testis and POF ovary can be exploited to regenerate the non-functional gonad. We had envisaged that it is the compromised niche that does not allow the surviving VSELs to restore gonadal function after oncotherapy. Based on this, Anand *et al.* (18) restored spermatogenesis and sperm production in busulphan treated testis by transplanting niche (Sertoli or bone marrow derived mesenchymal) cells via inter-tubular route into the testicular interstitium.

A critique of the study (1) is that they did not observe mES cells giving rise to SSCs in culture. Rather the mES cells differentiated into PGCLCs which directly underwent meiosis to produce haploid spermatids. In contrast, we have recently reported for the first time that small-sized VSELs, that survive busulphan treatment in mouse testis, undergo asymmetric cell divisions to give rise to slightly bigger SSCs (22). A chemoablated testis serves as an excellent model to study stem cells biology. The SSCs in turn divide rapidly by symmetric cell divisions followed by clonal expansion to form cell aggregates (*Figure 1*). We had earlier shown that the testicular cells isolated from chemoablated testis on culture spontaneously differentiate into sperm (12). The Sertoli cells attach to the culture surface and provide a somatic support and act as a source of growth factors and cytokines required for stem cells differentiation. Conditioned medium collected from healthy Sertoli cells culture was used for the cultures supplemented with FSH (5 IU). Surprisingly no additional factors like activin or retinoic acid were added to induce the cells to undergo meiosis. At the end of 3 weeks culture we observed various stages of spermiogenesis, cytoplasmic drops being shed and appearance of mouse sperm with characteristic hook shaped head (*Figure 1*). On D7 we could detect Gfra, Dazl, Prohibitin and Protamine suggestive of presence of spermatogonial stem cells, germ cells, spermatocytes and sperm in culture and that the cells underwent meiosis resulting in expression of post-meiotic markers.

But our work was published in an Open Access journal (12) compared to *Cell Stem Cell* which provided a home to Zhou and co-workers' study (1). Evidently, this difference is because they followed the gold standards proposed for *in vitro* derivation of germ cells (23). One needs to deliberate what was better, obtaining haploid spermatids or a large number of sperm showing all stages of stem cells biology (asymmetric cell divisions, symmetric cell divisions, clonal expansion) followed by spermatogenesis in a culture dish! The sperm generated *in vitro* were not tested further since we are apprehensive of sperm obtained in a dish from VSELs after 21 days culture for clinical use. We are more enthusiastic to restore spermatogenesis by endogenous manipulation of surviving stem cells in a non-functional, chemoablated gonad by injecting healthy niche cells (17) and allow Mother Nature to do the rest. Sperm collected from the caudal epithelium part of epididymis by this approach were able to fertilize eggs *in vitro* and initiate cleavage. Several groups have observed similar success and live births in mice on transplanting mesenchymal cells in both non-functional testis and ovary (24). A human baby

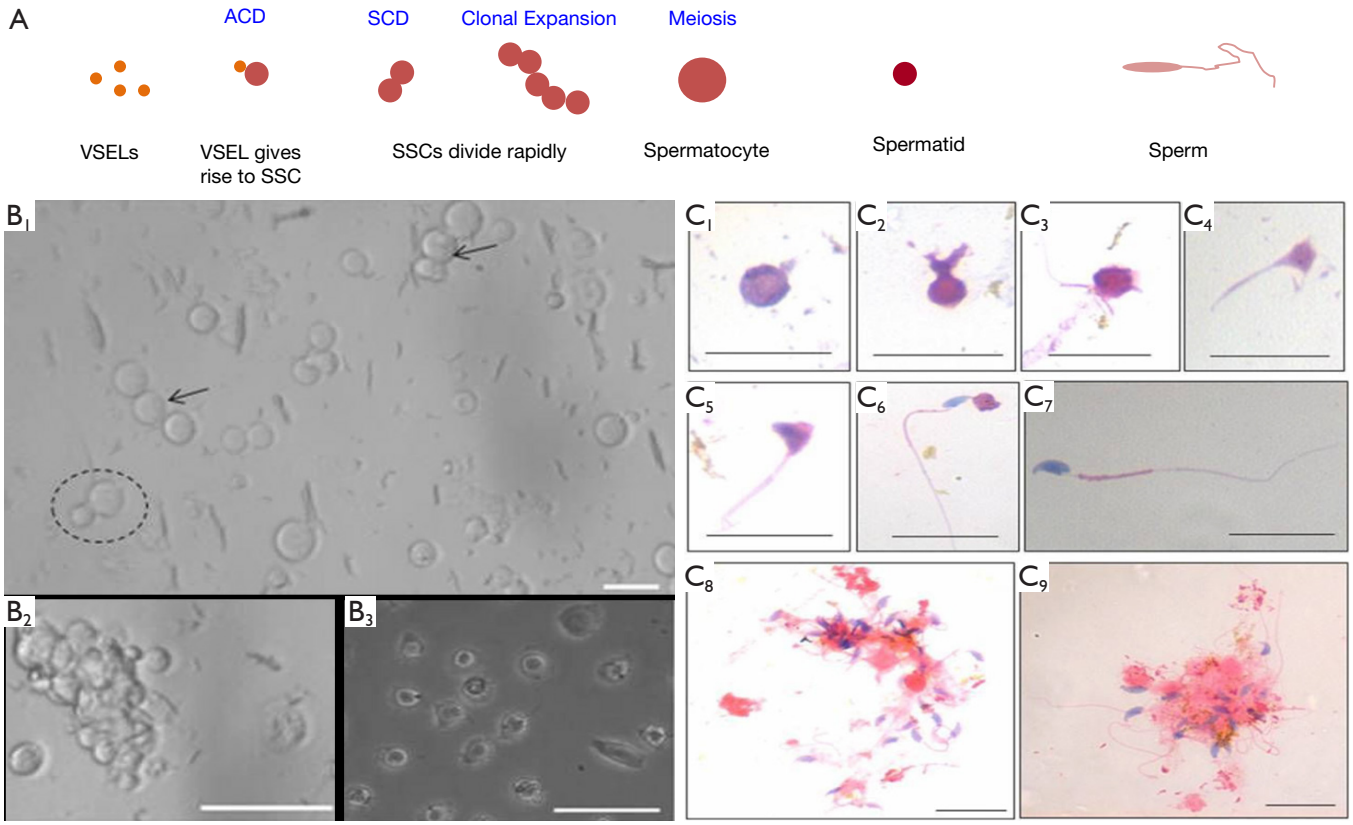

Figure 1 Stem cells survive chemotherapy and can restore spermatogenesis. (A) Stem cells biology of testicular VSELs and SSCs. VSELs are spherical cells of small size (3–5 µm) which give rise to SSCs (>5 µm) by asymmetric cell division (ACD). SSCs divide by symmetric cell division (SCD) and also undergo clonal expansion implying rapid division with incomplete cytokinesis. Spermatocytes then undergo meiosis to produce spermatids which undergo further spermiogenesis resulting in sperm. Similar ACD, SCD and clonal expansion was reported in testicular cell smears, for the first time by Patel and Bhartiya (22) on treating chemoablated mouse with follicle stimulating hormone (10 IU). (B_1) Cells separated from busulphan treated mouse testis show both ACD (broken circle) and SCD (arrow) after 24 h in culture whereas the Sertoli cells attach at the bottom as a feeder support to facilitate differentiation of stem cells. (B_2) Stem cells undergo clonal expansion by 3 days in culture to form clusters wherein cells retain cytoplasmic connectivity. (B_3) Large numbers of spermatids are observed in culture by 2 weeks. (C) H&E stained cells undergoing various stages of sperm differentiation from spermatids after 3 weeks culture. Various stages of spermiogenesis were evident. Round spermatocytes with protruding tail, spermatid, elongated spermatid with prominent tail, with residual cytoplasmic bodies, fully mature sperm with a long tail, hook shaped head and prominent mid-piece were observed. (C_8,C_9) At places sperm were observed in clusters with their heads embedded in residual cytoplasmic body. Bar =10 µm. This work has been published earlier by Anand *et al.* (12).

has been born by transplanting autologus mesenchymal cells in a POF ovary (25).

A critique to the study (12) was that all testicular cells that survived chemotherapy were used for *in vitro* culture. It could possibly be the few surviving SSCs that may have given rise to sperm *in vitro* rather than the VSELs. Whichever the starting cells (VSELs or SSCs)—at least a huge success was demonstrated for the first time *in vitro*! VSELs will invariably give rise to SSCs that will further differentiate. VSELs and SSCs are not two distinct stem cell populations, rather VSELs are the stem cells and SSCs are the progenitors (26). Indeed VSELs are the 'true' and most primitive stem cells in the testis which give rise to SSCs by asymmetric cell division (12,22). But criticism is always good and to further satisfy the reviewers, we have now studied differentiation of mouse bone marrow derived VSELs (no chance of contaminating SSCs) on a testicular niche and observed they successfully give rise to SSCs and

germ cells *in vitro* (personal observations).

We were intrigued to observe that PGCs were recently defined 'unipotent' in *Nature* journal (27) on the assumption that they only give rise to gametes in the body compared to totipotent or pluripotent early stage embryo/ICM which can form the whole body. This needs further clarification since like the ICM cells; even the PGCs express nuclear OCT-4 (28,29)—a transcription factor crucial to maintain pluripotent state. Loss of OCT-4 results in loss of PGCs rather than their differentiation into trophectoderm (28) suggesting an important role of nuclear OCT-4 in PGCs similar to in the ICM. In epiblast stage embryo, OCT-4 expression gets restricted to PGCs whereas the cells of all other cell lineages do no longer express OCT-4 (30,31). Similarly, conditional knockdown of Nanog (32) and SOX2 (33) also induced apoptosis and decreased numbers of migrating PGCs rather than their differentiation. Thus OCT-4, NANOG and SOX2 are crucial for PGCs; form the triumvirate to define pluripotent state (34) and their expression in PGCs reflects pluripotent state of PGCs.

VSELs are considered equivalent to PGCs and are indeed pluripotent both *in situ* as well as *in vitro*. They survive in all adult body organs (not restricted only to the gonads) and serve as a backup pool for various tissue specific stem/ progenitor cells throughout life (35,36). Also besides expressing pluripotent and PGC-specific markers, VSELs obtained from both mouse bone marrow [(37) and personal observations] and human cord blood (38) have the ability to differentiate into 3 germ layers *in vitro*. Scientific community needs to be convinced about the existence of VSELs and their potential. This will require revision of currently held views on basic property of PGCs (from unipotent to pluripotent) and also a re-look at the definition of the term 'pluripotency' (39). The deleterious effect of busulphan treatment on testicular Sertoli cells by microarray analysis and the underlying mechanism how transplantation of healthy niche (Sertoli or bone marrow mesenchymal cells) can restore spermatogenesis from endogenous VSELs in chemoablated testis was recently published (40). To conclude, endogenous VSELs are possibly better stem cell candidates to make gametes!

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Footnote

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