Special Article

From Embryonic Stem Cells to Functioning Germ Cells: Science, Clinical and Ethical Perspectives

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Embryonic stem cells have been well recognized as cells having a versatile potential to differentiate into all types of cells in the body including germ cells. There are many research studies focusing on the differentiation processes and protocols to derive various types of somatic cells from embryonic stem cells. However, germ cells have unique differentiation process and developmental pathway compared with somatic cells. Consequently, they will require different differentiation protocols and special culture techniques. More understanding and established in vitro systems for gametogenesis will greatly contribute to further progression of knowledge and technology in germ cell biology, reproductive biology and reproductive medicine. Moreover, if oocytes can be efficiently produced in vitro, this will play an important role on progression in nuclear transfer and nuclear reprogramming technology. The present article will provide concise review on past important discoveries, current ongoing studies and future views of this challenging research area. An ethical perspective has also been proposed to give comprehensive summary and viewpoint for future clinical application.

Keywords: *Embryonic stem cell, Germ cell, Sperm, Oocyte, In vitro, Gametogenesis, Ethics*

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Embryonic stem cells (ESCs) have been well recognized as cells having a potential to differentiate into all types of cells in the body. They are promising therapeutic tools for cell and tissue therapies. There are many research studies focusing on differentiation processes and protocols to derive various types of somatic cells from embryonic stem cells. The greatest attention results from the high demand and high prevalence of cardiac diseases and degenerative nervous system disorders. Moreover, stem cell-based tissue replacement therapy is also a promising approach for plenty of incurable and untreatable conditions.

However, germ cells also have a significant role on basic knowledge and in-depth understanding in developmental and stem cell biology because oocytes and sperm have unique differentiation process and developmental pathway when compared with somatic cells. Meiotic division and imprinting are unique key point steps to convey genetic material and biological information from generation to generation[1-3]. Germ cell derivation undoubtedly requires different differentiation protocols and special culture techniques. More understanding and established *in vitro* system for gametogenesis will greatly contribute to further progression of knowledge and technology in germ cell biology, reproductive biology and reproductive medicine. Furthermore, if oocytes can be efficiently produced *in vitro*, this will play an important step forward on progression in nuclear transfer and nuclear reprogramming technology[1-3].

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Review of previous important discoveries

The first landmark paper for *in vitro* germ cell derivation from mouse embryonic stem cells was reported from Hubner et al[4] in Science (2003). With specially constructed germ cell specific OCT4-Green Fluorescent Protein (gcOCT4-GFP) reporter, they could sort and culture germ cell specific embryonic stem cell

lines in monolayer culture system. These cells spontaneously differentiated into follicle-like structures within 26 days. The hormonal production capacity of the constructed follicle was demonstrated by estradiol assay. Estradiol production increased when follicle-like structures have been cultured for a longer period of time beginning at 12 days and reaching its maximal level in 20 days. This estradiol production well reflects the hormonal function of follicle-like unit in the culture medium. In the present study, the estradiol production showed the hormonal competency of suspected granulosa cells and their companion theca cells. More details in progressive expression of key genes required for estradiol production-steroidogenic acute regulatory protein (StAR), P450 17alpha-hydroxylase-17/20 lyase (CYP17), and P450 aromatase-have been clearly demonstrated. In addition to estradiol production, follicle-like structures have been tested for evidence of meiosis process. Synaptonemal complex protein (SCP) 3, well-recognized important marker for meiosis, can be detected in these structures. As a result, they proposed that these putative oocytes have already entered into meiotic division. For reproductive function of oocyte-like structure, there is still no evidence or experiment performed to test for their fertilization capability. However, after 43 days in culture medium, they found blastocyst-like structure. The formation of these blastocyst-like structures was probably caused by parthenogenesis. However, more in-depth details on the blastocyst formation have not been characterized and reported.

The second report for *in vitro* gametogenesis was from Japan. Toyooka et al[5] showed their findings in PNAS (2003). This study used a different reporter system. Instead of gcOCT4-GFP, this group traced germ cell formation with the mouse vasa homologue (Mvh) gene tagged with GFP. Mvh is a germ-cell-specific ATP-dependent RNA helicase not expressed in ESCs and consequently enables the tracking of germ cell differentiation in culture. ESCs were aggregated into three dimension (3D) structures, embryoid bodies (EBs), comparing with monolayer culture system used in the first report. Putative germ cells were then fluorescence-activated cell sorted (FACS) by GFP expression. Afterward, these cells were co-cultured with gonadal cells, and transplanted into a testicular capsule. Mature sperm has been formed. However, demonstration of an ESC origin of sperm by donor-specific markers was lacking and functional data concerning the fertilization capacity of these ESC-derived sperm were not provided. In this study, they have also identified that bone morphogenetic protein 4 (BMP4) play an important role on *in vitro* gametogenesis. Nevertheless, BMP4 action on germ cell development *in vitro* is quite complicated. An inductive effect of BMP4 occurred only when the ES cells were coaggregated with the BMP4-producing cells; no induction was found when BMP4 was added to a suspension culture of ES cells or when ES cells were cultured in tissue culture dishes with BMP4-producing cells as feeder cells. Therefore, it seems that the production of germ cells from ES cells depends on the constant stimulation of BMP4, together with a unique three dimensional configuration of the EBs. They could produce a proper community, appropriate "niche", effect mimicking cell to cell interactions *in vivo*.

One year later, Geijsen et al[6] reported their findings in Nature (2004). With EB culture system after ESCs had undergone differentiation for 4-10 days, cells were sorted with the surface marker stage specific embryonic antigen 1 (SSEA1), which is expressed by both germ cells and ESCs. To isolate the clonal lines of embryonic germ cells (EGCs), ESCs were cultured in medium enriched with retinoic acid (RA) and germ cell growth factors - beta fibroblast growth factor (bFGF) and stem cell factor (SCF) - which stimulated residual ESC differentiation and promoted germ cell self-renewal. These *in vitro* derived germ cells exhibited correct imprinting pattern, which is one hallmark of normal *in vivo* germ cell development. If continued culturing these cells in the EBs for longer time periods, more mature round spermatid-like cells developed. They were then sorted out and isolated using an antibody for the sperm acrosome (FE-J1). Purified cell population, which was mostly haploid cells, was injected into oocyte, can complete fertilization leading to the formation of blastocyst. However, no live offspring has been reported to date with this sperm-like cell.

In 2006, there were two important reports on oocyte derivation from mouse ESCs. Lacham-Kaplan et al[7] have shown that growth factors in conditioned medium collected from testicular cell cultures prepared from the testes of newborn males repeatedly and reliably supported development of ESCs into follicle-like structures, which contained putative oocytes. Their oocytes were surrounded by one to two layers of flattened cells and did not have a visible zona pellucida. However, oocyte-specific markers such as factor in the germline alpha (Fig-alpha) and zona pellucida 3 (ZP3) were found expressed by these ovarian structures. Novak et al[8] have also made further progression on details of meiosis process for *in vitro* oocyte

development. They also used EB culture system and cocultured ESCs with BMP4 producing cell line as previously described by Toyooka et al. They have carefully evaluated the meiotic process in germ cell-like cells derived from ESCs, using a panel of meiosis specific markers that identify distinct meiotic signatures unique to meiotic prophase I development *in vivo*. Whereas synaptonemal complex protein (SCP) 3 is expressed in germ cell-like cells, other meiotic proteins, such as SCP1, SCP2, stromal antigen 3 (STAG3), REC8 (meiotic protein similar to the rad21 cohesins), and structural maintenance of chromosomes-1 (SMC1)-beta, are not expressed. The nuclear distribution of SCP3 in the germ cell-like cells is also highly abnormal and not associated with the chromosomes of these cells. They further performed fluorescence in situ hybridization analysis showing that the SCP3-positive germ cell-like cells do not contain synapsed homologous chromosomes but instead display a chromosomal organization normally found in somatic cells. The absence of expression of essential meiotic proteins and a normal meiotic chromosomal organization in this study strongly suggests that the germ cell-like cells formed from ESCs fail to progress through meiosis. To date, no pups have been created from oocyte derived from ESCs.

For *in vitro* male gametogenesis from ESCs, a significant break through in this interesting area has been contributed by Nayernia et al[9]. They have developed a new strategy for the establishment of spermatogonial stem cells (SSC) lines from embryonic stem cells (ESCs). Retinoic acid is a key substance in this culture system. These cell lines are able to undergo meiosis, are able to generate haploid male gametes *in vitro*, and are functional, as proved by successful fertilization after intracytoplasmic injection into mouse oocytes. Resulting two-cell embryos were transferred into oviducts, and live mice were born. Six of seven animals had potential to develop into adult mice. Result from this study is the clear evidence that male gametes derived *in vitro* from ESCs are capable of inducing normal fertilization and development.

For *in vitro* germ cell formation from human ESCs, a first significant discovery has been reported from Clark et al[10]. They provided the first evidence that human ESCs can differentiate into germ cells. EBs were again used to create the proper environment for germ cell formation *in vitro*. This study provided a detailed transcriptional profile of EBs that suggested the onset of both immature germ cells and more mature gonocytes. In addition, markers for both female and male germ cells were found regardless of the sex of the ESC lines. Although genes involved in meiosis were also detected, no isolation of haploid gamete cells was achieved. Whereas this study shows the possibility of germ cell development from human ESCs, further works is still required to identify, characterize and, most importantly, isolate these putative germ cells and test the reproductive capability of them.

Current ongoing studies

The goal of most current studies in this challenging area is to establish reproducible and efficient protocols to derive competent germ cells from ESCs. From previous findings, knowledge and techniques for male gamete formation seems to progress more rapidly than female gamete. *In vitro* derived sperm has been shown to produce live offspring already while currently there is no live pup born from putative female germ cells derived from ESCs.

Parallel experiments and studies have been carried out on human ESCs with a purpose to apply this knowledge and technique to use in clinical practice finally. However, more in-depth knowledge and studies on human germ cell differentiation and development are required due to differences in mouse and human germ cell development. Mouse genetics and early germ cell development *in vivo* have been thoroughly studied and characterized. Unfortunately, for early germ cell studies in human embryos and fetus, *in vivo* experiments are impractical and nearly impossible. As a result, *in vitro* model for gametogenesis will be a very useful tool for studying human germ cell biology.

Future trends

In addition to germ cell knowledge, *in vitro* gametogenesis will provide a good model to study germ cell niche. Furthermore, this is also a very useful model to trace differentiation and developmental process of somatic cells (e.g. granulosa and theca cells) supporting gamete formation and interaction between these cells within follicle unit.

The scope of *in vitro* germ cell derivation will not focus only on germ cell biology and reproductive biology. Because of the requirement of a number of oocytes for nuclear transfer and nuclear reprogramming experiments, efficient production of oocytes in laboratory will have a high impact on progression of this core knowledge and technology in stem cell biology. If human oocytes can be easily produced from human ESCs *in vitro*, medical risk for oocyte donors from ovarian hyperstimulation syndrome could be avoided

and there might be fewer ethical problems on the issue of oocyte donation.

Clinical applications of embryonic stem cell derived germ cells

The most prominent potential use of *in vitro* germ cell derivation in the clinic is for infertile couples. Combined with nuclear transfer technology, gamete may be produced from somatic cells (e.g. skin cells), infertile patients could have a new option to have children with their own genetic material. This will solve the problem of scanty of gamete donors and the sensitive issue for obtaining gametes donated by others.

The other often-overlooked clinical importance of *in vitro* gametogenesis model is the capability to create ovarian somatic cells in the laboratory. These supporting (granulosa and theca) cells will be a very useful source of hormonal production for cases with inability or insufficiency in sex hormone production both innate and acquired from diseases or medical treatment and/or surgery.

Ethical consideration

In vitro gametogenesis from ESCs could provide a limitless resource for both research and medical treatment. *In vitro* germ cell production may have less medical risk for donors during the process of ovarian stimulation and oocyte collection. However, there are still many ethical issues to be carefully considered before applying this promising knowledge into medical practice.

Testa and Harris[11,12] have proposed that, with gametes produced from ESCs formerly derived by somatic cell nuclear transfer (SCNT), it is possible that same-sex couples could have offspring with genetic contributions from both partners. This will bring up many issues on health risks of future offspring, including social and ethical concerns. It may be also be the issue of equity and justice for heterosexual and homosexual couples to have children with their own genetic material.

Another issue raised is the human status and dignity of a person born from gametes derived from ESCs. It is unclear how these children will feel about their status and their sense of human beings[11-14]. This may or may not be like the case of children born from *in vitro* fertization (IVF) since they may not have a father who provided sperm and a mother who provided an egg. There is an opinion that prospective parents who take the burden and cost of medical intervention to procreate are clearly demonstrating a responsibility and commitment to their offspring that is arguably stronger, for example, than in the case of unwanted pregnancies. Infertile heterosexual couples and homosexual couples who reproduce, now through gamete donation and surrogacy, and one day possibly through ESCs derived germ cells, are asserting a responsibility toward entities that they are unlikely to view as only "assemblages." The viewpoint that this responsibility is not "socially prescribed" for homosexual couples may or may not be considered as discrimination and inequity.

Conclusion

Knowledge and technology involved with *in vitro* germ cell derivation from ESCs will have a great impact on understanding and the progression in germ cell biology, reproductive biology, and reproductive medicine. This will also provide essential resources for somatic cell nuclear transfer and nuclear reprogramming studies, which are currently the highlight topic in stem cell biology. However, there are many technical and ethical issues to be considered. Finding appropriate solutions before transferring this challenging advance into clinical practice is required.

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จากเซลล์ต้นกำเนิดตัวอ่อนสู่เซลล์สืบพันธุ์: มุมมองทางวิทยาศาสตร์ คลินิก และจริยธรรม

สรภพ เกียรติพงษ์สาร

เซลล์ต้นกำเนิดตัวอ่อนได้รับการยอมรับถึงการมีศักยภาพสูงในการพัฒนาไปเป็นเซลล์ทุกชนิดในร่างกาย รวมถึงเซลล์สืบพันธุ์ได้ มีการวิจัยจำนวนมากที่มุ่งเน้นศึกษาถึงกระบวนการพัฒนาและค้นคว้าหาสูตรวิธีการสร้างเซลล์ร่างกายหลากหลายชนิดจากเซลล์ต้นกำเนิดตัวอ่อน หากแต่เซลล์สืบพันธุ์นั้นมีกระบวนการพัฒนาและขั้นตอนการเจริญเฉพาะต่างจากเซลล์ร่างกายโดยทั่วไป จึงต้องการสูตรวิธีการสร้างพัฒนาเซลล์และวิธีการเพาะเลี้ยงเซลล์แบบพิเศษ ความเข้าใจที่มากขึ้นและความสามารถในการสร้างเซลล์สืบพันธุ์ขึ้นได้ในหลอดทดลองจะช่วยให้เกิดความก้าวหน้าทางความรู้และเทคโนโลยีด้านชีววิทยาเซลล์สืบพันธุ์ ชีววิทยาการเจริญพันธุ์ และเวชศาสตร์การเจริญพันธุ์ อีกทั้งหากสามารถสร้างเซลล์ไข่ขึ้นได้ภายในห้องปฏิบัติการอย่างมีประสิทธิภาพ ก็จะเป็นปัจจัยช่วยสำคัญต่อความก้าวหน้าในเทคโนโลยีการเปลี่ยนถ่ายนิวเคลียสระหว่างเซลล์และการเปลี่ยนย้อนระบบการทำงานภายในเซลล์ บทความนี้มีเนื้อหาทบทวนการค้นพบที่สำคัญในอดีต สรุปงานวิจัยที่กำลังอยู่ในระหว่างดำเนินการและแนวทางการศึกษาวิจัยในอนาคต นอกจากนี้ได้มีการนำเสนอแนวคิดและประเด็นด้านจริยธรรมที่เกี่ยวข้องเพื่อให้เกิดมุมมองที่ครอบคลุม บูรณาการให้เห็นถึงภาพรวมและเกิดแนวคิดต่อการนำไปประยุกต์ใช้ในทางคลินิกในอนาคต