Studying Spermatogenesis by using In vivo and In vitro Models: Advantages and Disadvantages of these Models for Practical Use
Yoko Sato, Masayasu Taniguchi and Takeshige Otoi*

Laboratory of Animal Reproduction, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1, Yoshida, Yamaguchi 753-8515, Japan

Abstract

Several experimental systems are available for inducing spermatogenesis outside the endogenous testis. These systems have been developed as tools for studying spermatogenesis and as an option for preserving genetic material obtained from males when sperm recovery is not possible. Two in vitro systems are available for this purpose: tissue grafting and cell transplantation. Ectopic grafting of immature testicular tissues into immunodeficient mouse hosts is a type of in vivo system that allows the immature testicular tissue from many types of animals to undergo complete spermatogenesis. The other in vivo system is germ cell transplantation into the recipient testis, which induces colonization of spermatogonial stem cells from many types of animals and allows the stem cells to differentiate into spermatocytes in some cases. Furthermore, 2 in vitro systems are available: tissue culture and 3-dimensional (3D) cell culture. The tissue culture system and the combination of tissue culture and germ cell transplantation system were developed recently; this made it possible to perform complete spermatogenesis by using mouse spermatogonial stem cells. Isolated immature mouse testicular cells can differentiate into spermatocytes when the 3D culture system is used. All these systems have advantages and disadvantages with respect to studying spermatogenesis and preserving fertility in many types of animals. Therefore, it is necessary to consider many factors that might affect the results of spermatogenesis in order to use these experimental systems appropriately. Herein, we have discussed the advantages and disadvantages of these systems, especially in connection with several factors that may affect spermatogenesis.

Keywords: Spermatogenesis; Tissue grafting; Germ cell transplantation; Tissue culture; 3D germ cell culture

Introduction

Spermatogenesis is a complicated process consisting of a proliferative stage, meiotic stages, and differentiation or spermiogenic stage [1]. The process of spermatogenesis continues throughout most of adulthood in mammals. Complete differentiation of the spermatocytes requires more than 1 month in most mammals.

Many experimental animal models are available for analyzing the process of spermatogenesis, including transgenic animals and strains that inherently lack spermatogenesis [2,3]. In contrast, several experimental systems for inducing spermatogenesis in vitro or in vivo have only been developed in recent decades [4-10] for use as tools for studying the fundamental aspects of spermatogenesis and as an option for preserving genetic material obtained from males when sperm recovery is impossible, for example, from rare and endangered species [11] and immature cancer patients [12]. Furthermore, these systems are useful for studying toxic or irradiation effects on germ cells.

Herein, we introduce in vivo systems that use tissue grafting and cell transplantation and in vitro systems that use tissue culture and 3-dimensional (3D) cell culture. All these systems have advantages and disadvantages with respect to studying spermatogenesis and preserving fertility in many types of animals. Many factors can affect the results of spermatogenesis when these systems are used. In this review, we have introduced and summarized several factors that may affect spermatogenesis (Table 1).

Factors Affecting Spermatogenesis Using In vivo Systems

Grafting

The greatest advantage of the grafting method is the ability to induce complete spermatogenesis by using immature testicular tissue from different mammalian species in fresh or cryopreserved conditions. Furthermore, spermatogenesis can be accelerated in the graft. However, this method is not adequate for analyzing cell-to-cell interactions. Furthermore, it has a limitation with respect to controlling the environmental conditions of the grafting tissue because of the use of an in vivo system.

Donor age: Xenografting of testicular tissue from immature males to immuno-deficient mouse hosts results in germ cell differentiation and production of sperm from mammalian species such as pigs [7], goats [7], hamsters [13], rabbits [14], bulls [15], rhesus monkeys [16], cats [17], and horses [18], but not from marmosets [19] or humans [20]. In the case of mouse, pig and rabbit donors, the spermatogenesis produced in the grafted tissue show fertilization competency [7,14,21]. However, xenografts from sexually mature animals cannot survive for more than 12 weeks, and most of the seminiferous tubules in the grafts show degeneration in pigs, goats, and cattle [22]. In contrast, xenografted testicular tissue from young adult (3-year-old) rhesus monkeys donors have been reported to survive better than grafted tissue from other older adult donors and show complete spermatogenesis, although this is species-specific, for example, xenografted testicular tissue from young adult donors of pigs and goats do not improve the results [22]. Therefore, it is better to use immature tissue for grafting when a more

*Corresponding author: Takeshige Otoi, Laboratory of Animal Reproduction, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1, Yoshida, Yamaguchi 753-8515, Japan, Fax: +81-83-933-5904; E-mail: otoi@yamaguchi-u.ac.jp

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advanced stage of spermatogenic cells is required. However, it may be possible to obtain spermatogenic cells at an advanced stage from adult grafts.

The reasons for poor survival and differentiation of adult testicular tissue in xenografts are so far unknown. However, the developmental age of the grafts at the time of transplantation may be responsible. The grafting tissue is subjected to ischemic conditions from the beginning of the procedure until angiogenesis established between the graft and the recipient animal. The circulatory connections between the graft and host are established by a combination of outgrowth of small capillaries from the donor tissue and formation of larger vessels by the host [23]. Different types of germ cells may have different sensitivities to hypoxia during spermatogenesis. For example, the metabolism of round spermatids in rats exclusively depends on oxygen [24], which may result in the survival of spermatogonia but not round spermatids in the adult testicular graft. Because mature material has a higher sensitivity to ischemia than immature material does [18,25], grafting adult tissue from mature animals may have low efficiency for preserving spermatogenesis. Pretreatment of testicular grafts with vascular endothelial growth factor in order to improve angiogenesis in the grafted tissue results in improved germ cell differentiation in xenografts of immature bovine testicular tissue [26]. This treatment may be useful for grafting adult testicular tissue from other animals. Recently, Li et al. [27] reported successful maintenance of spermatogenesis by revascularized orthotopic adult testicular transplantation in mice, confirming that angiogenesis is important for the graft. Introducing vasculature between the graft and recipient animals as soon as possible after grafting might help to induce more advanced spermatogenesis in the adult graft.

However, the developmental age of the grafts at the time of transplantation may not be the only factor that affects grafting results. Morphological analysis of infant and adult testicular tissues has shown more complicated structure in adult tissues than in immature tissues; that is, adult testicular tissues contain many more advanced stages of germ cells over spermatogonia than immature tissues [1]. Therefore, there are several trials to use the adult tissue showing suppression of spermatogenesis for grafting. The survival and spermatogenic efficiencies of xenografts are much higher during xenografting of cryptorchid tissue that lacks spermatogenesis at the time of grafting than during xenografting of normal adult donor tissue with full spermatogenesis at the time of grafting, while using horses and mice as recipients [28,29]. Furthermore, human adult testicular tissue from patients with suppressed spermatogenesis show better survival as xenografts than tissue from donors with complete spermatogenesis at the time of grafting [28]. GnRH antagonist treatment of donor testes obtained from adult mice showing suppressed spermatogenesis before grafting showed enhanced survival of spermatogenic cells and differentiation until elongated spermatid [30]. Although adult photoregressed hamster testicular tissues partially recover function after grafting, they exhibited degenerate tissue frequently [31]. Using adult tissue with suppressed spermatogenesis might be an option for use in adult testis tissue grafting to induce differentiation of spermatogonial stem cells (SSCs) into more advanced stages; however, this procedure requires improvements.

These reports suggest that spermatogenic differentiation is dependent on the age of donor as well as the degree of spermatogenesis in the tissue at the time of grafting, even if there is a species-specific difference.

**Donor tissue storage:** Cryopreservation is a useful method for maintaining functional (SSCs) from mice and rabbits [31]. Combining testis cryopreservation with grafting procedure may be a powerful tool for restoration of fertility, especially for immature animals and prepubertal patients.

Cryopreservation of tissue does not have an obviously unfavorable effect on spermatogenesis in testicular tissue grafts from neonatal and adult mice [31] when Dimethylsulfoxide (DMSO) is used as a cryoprotectant. Spermatogonia can also survive and proliferate after cryopreservation and orthotopic xenografting of immature human cryptorchid testicular tissue from young boys [32]. However, progression of spermatogenesis to round spermatids has not been

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| Grafting | Transplantation | Tissue Culture | 3D in vitro culture |
|----------|-----------------|----------------|---------------------|
| Donor age | (7,13,14,15,16,17,18,19,20) | - (62) | ND | ND |
| Donor tissue or cell preservation | + (34,33) | + (31) | - (8,9) | ND |
| Cross species | + (19) | + (54,55,56,57,58) | ND | ND |
| Recipient location | subcutaneous- (7,13,14,15,16,17,18,19,20) | + (71) | ND | ND |
| Recipient treatment | - (38,46) | + (85,86,53) | +(combi) (9) | ND |
| Recipient age | + (39) | + (62) | ND | ND |
| Others | acceleration of spermatogenesis | decrease in litter size (88,89) | only mouse data (8,9) | only mouse data (10) |

*: positive effects on spermatogenesis; -: no effects on spermatogenesis; ND: no data; combi: combination of tissue culture and germ cell transplantation; 3D: 3D culture

Table 1: Factors that affect spermatogenesis in experimental systems.
achieved for sperm in xenografts from cryopreserved immature porcine testes [33]. Furthermore, Jahnukainen et al. [34] reported that cryopreservation delays the initiation of spermatogenesis in the grafted tissue of juvenile rhesus monkey testicular grafts because it affects either the number of surviving type A spermatogonia or their capacity to colonize the seminiferous tubules. Although tissue cryopreservation in 1.4M DMSO allowed the rhesus monkey graft to initiate spermatogenesis, 0.7 M DMSO and ethylene glycol provided lower protection, suggesting that both the type and dose of the cryoprotective agent are critical for graft survival [34] and that the effects of cryopreservation depend on the species [13,33]. Species-specific morphological differences in the walls of seminiferous tubules, including the structure of the lamina propria [35] and in the stroma, including Leydig cells, blood, and lymph vessels [36], may cause variation in the duration of cryoprotectant permeation into various cells of the tissue and in the efficacies of the cryoprotectants.

However, cooling to 4°C for 24 h before xenografting appears to further improve the survival of rhesus monkey testicular tissue or the capacity of SSCs to colonize or initiate spermatogenesis [34]. Furthermore, complete spermatogenesis occurs in porcine xenografts preserved by cooling at 4°C up to 48 h [33]. Under cooling conditions, exposure to ischemia for at least 1 or 2 days does not appear to affect the grafting results of immature rhesus monkey testis and porcine testis, respectively.

Thus, the necessity for optimizing cryopreservation conditions for each kind of animal tissue should be taken into consideration for grafting. Furthermore, cooling the tissue at 4°C might be considered as an option for short-term storage.

**Endocrinological factors:** Castration is thought to be essential for the development of xenografts in the recipient mouse. Removal of the testes results in a lack of androgens, allowing the serum levels of gonadotropins to increase. Thus, the increased serum levels of LH and FSH immediately after castration were thought to induce the same hormonal conditions for immature testis xenografts as in puberty, without the release of sufficient testosterone, thereby stimulating the proliferation and differentiation of spermatogenic cells [37]. Within 2 weeks after grafting, immature testis grafts release enough testosterone to establish feedback on gonadotropin release in the recipient mouse [7,37]. However, it was recently reported that gonadectomy had no evident effect on the outcomes of porcine tissue xenografting; furthermore, xenografts in female recipient mice with intact ovaries did not show any difference between the host strains [18]. However, NOG mice (NOD/Shi-SCID, IL-2Rγ null), which are more severely immunocompromised than nude and SCID mice [42], do not show the most successful development of porcine spermatogenesis among host strains [41]. These reports suggest that a hospitable microenvironment has little effect on the immunocompetency of the recipients, depending on the recipient species.

**Acceleration of spermatogenesis in the graft:** The period required for differentiation of spermatogenic cells in immature testicular xenografts differs depending on the animal species used as a donor source. Compared with the rate of spermatogenesis in the donor species, the rate of spermatogenesis is accelerated in testicular xenografts from pigs [7], sheep [7], rhesus monkeys [16] and humans [20] but not in those from cats [17] or cattle [15,43]. The reason for the shortened time to differentiation in the xenografts is not yet known. However, this characteristic might be practically useful for performing experiments on animals that require a longer period for spermatogenesis.

**Grafting of isolated testicular cells:** Isolated testicular cells that have been obtained from piglets and have been enzymatically digested can regenerate complete testis tissue after implantation [44]. In the case of mice and rats, testicular cells in the reconstituted testis of the graft could differentiate into fertilization-competent round spermatids [45]. The reconstitution of seminiferous tubules from neonatal testicular tissue of various mammalian species may be possible. Furthermore, manipulation of specific pathways in germ cells or somatic cells before re-aggregation will provide a controlled accessible system for studying cell-to-cell interactions governing testicular morphogenesis and spermatogenesis.

**Autologous, heterologous, or xenologous transplantation in marmosets:** As mentioned in the "Donor age" section, xenografting of testicular tissue from immature marmosets to immunodeficient mouse hosts does not result in germ cell differentiation and sperm production as seen for some other mammalian species [19,46]. However, orthotopic immature testicular tissue grafts show complete spermatogenesis during autologous grafting in marmosets, although the grafts show spermatogenesis arrest during xenologous grafting in immunodeficient mice [47].

Spermatogenesis failure in marmoset testicular tissue xenografted in immunodeficient mice was initially thought to be caused by the differing functions of the LH/chorionic gonadotropin (CG) system in the 2 species, which is also found in other neotropical monkeys [48,49]. Because the mice did not express CG, the host endocrine environment, which involved factors such as CG and androgen, could not support testicular tissue development of the graft from the marmoset. Wistuba et al. [46] used immature hamster testicular tissue for co-grafting to create high local levels of testosterone release at the implantation sites of the marmoset graft; however, neither normal serum androgen levels nor the high local testosterone levels were sufficient to initiate marmoset spermatogenesis. Furthermore, administering hCG to the transplanted donor mouse did not rescue spermatogenic arrest. They suggested that initiation of marmoset spermatogenesis under xenologous conditions required factors more complicated than simply providing a hormonal milieu that was similar to the original conditions in the marmoset.

**Location of the transplantation site in marmosets:** The transplantation site has been shown to affect the maturation rate of marmoset testicular grafts [47,50], although spermatogenesis is
completed successfully in ectopic grafting of other species except marmoset. Autologous transplants of immature marmoset testicular tissue show complete spermatogenesis in orthotopic but not in ectopic conditions [47]. Thus, local factors might influence the differentiation occurring during spermatogenesis in the grafts at the different transplantation sites. In marmosets, the greatest difference between the locations is presumably local temperature because the marmoset’s back is covered with thick fur and the subcutaneous temperature at the back is almost 5°C higher than that at the scrotum [19]. These results suggest that the location of the transplantation site might need to be taken into consideration in the case of furry recipient animals.

Cell transplantation

Although cell transplantation does not cause adequate complete spermatogenesis in the case of cross-species transplants, except in rodents, this method can be used to induce proliferation of SSCs from different mammalian species in fresh or cryopreserved conditions. Compared to the grafting technique, the advantage of this technique is that it allows analysis of cell-to-cell interactions in the testicular tissue, although it is still difficult to observe spermatogenesis in real time. Furthermore, this method has a limitation with respect to controlling the environmental condition of the graft because it involves an in vivo system just like the grafting method.

Cross-species transplantation-related issues: Germ cell transplantation is a potential alternative approach for preservation and differentiation of spermatogenic cells [5,6]. Cross-species germ cell transplantation from rats to mice has been used to produce rat sperm in the mouse testis [51], and the reverse procedure has also succeeded [52,53]. However, spermatogenesis between different species has been successful only in rodents. Although transplanted non-rodent spermatogonia can colonize host immunodeficient mouse testes, differentiation has not been observed using rabbits [54], dogs [54], pigs [55], cattle [55], horses [55], baboons [56], or humans [57] as donors. Even in rodents, hamster spermatogonia show abnormal spermiogenesis after transplantation [58]. Honaramooz et al. reported that when goat germ cells were transplanted into an immunocompetent goat, complete spermatogenesis occurred, and the sperm exhibited fertilization competence [59].

The types of factors responsible for the difference in the effects of xenologous and autologous germ cell transplantation are unclear. With respect to spermatogonial proliferation after xenotransplantation, immunological inconsistency may not be the only cause of failure of spermatogenesis after allogenic transplantation in mouse [60]. In xenologous germ cell transplantation, germ cells must adapt to the new niche from the endogenous niche. The niche for spermatogonial proliferation appears to be generally similar among different species because proliferation is possible between cross-species after xenotransplantation of spermatogonia. However, the niche for spermatogonial differentiation is thought to have species-specific mechanisms because completion of germ cell differentiation has not been shown among distantly related species after xenotransplantation [19]. Sertoli cells partially support the formation of the niche for spermatogonia. One of the reasons for incomplete spermiogenesis in hamster spermatogonia after xenotransplantation may be the different distributions of glial cell line-derived neurotrophic factor (GDNF), a major Sertoli cell-derived factor regulating the maintenance of undifferentiated spermatogonia, in the Sertoli cells of mice and hamsters [61]. The successful transplantation of germ cells is thought to be positively related to the degree of evolutionary relatedness of species. Furthermore, if we can set the suitable niche for differentiation of spermatogonia on cross-species transplantation, it may be possible to produce the sperm under xenologous germ cell transplantation.

Donor age: Shinohara et al. [62] reported that the colonization area and extent of spermatogenesis at 2–3 months after transplantation did not differ among donor cells from neonate, immature, and cryptorchid adult testes, although the number of stem cells increased in the immature testis during normal postnatal development in vivo. This report suggests that the capabilities of the SSCs for both proliferation and spermatogenesis are uniform during development, at least with respect to transplantation. Because of this characteristic, SSCs transfer from various ages of the donor animal might be useful for examining the proliferation activity of SSCs.

Preservation of spermatogenic cells: Compared to the cryopreservation method used for spermatooza, this method used for spermatogenesis is simple and similar to those generally used for somatic cell lines. Cryopreserved testicular cells from animals such as immature or adult mice [63], calves [64], rabbits [54], dogs [54], pigs [55], cattle [55], horses [55], baboons [56], and humans [57] show SSC survival or proliferation when used for transplantation. Freeze-thawed mouse testicular cells possess higher stem cell colonization efficiency and fertilization ability than fresh donor cells [31], but cryopreservation of human testicular cells does not influence their stem cell colonization efficiency [57].

Cryopreservation of testicular tissue for cell transplantation has another benefit. The amount of stem cells used for infusion is an important factor affecting efficient sperm production after testicular cell transplantation [65]. Testicular SSCs can presumably survive at a higher rate than other testicular cells because SSCs have high resistance against a variety of agents that damage the testes, including irradiation and chemical insults [66,67]. The greater viability of the testicular SSCs may result in a larger population of these stem cells after cryopreservation than in fresh testes [31]. Thus, cryopreservation is useful for improving both maintenance and efficient transplantation of SSCs.

Infusion route: Germ cells can be transplanted into the testis via several routes, including microinjection into the seminiferous tubules, the efferent ducts, or the rete testis [68–70]. In the mouse testis, both microinjection in seminiferous tubules and cannulation in efferent ducts are useful and equally effective methods for transfer of germ cells [68,70]. Ultrasound-guided injection into the rete testis provides efficient germ cell transfer into the seminiferous tubules in bulls, monkeys, and humans [71].

With respect to infusion, better infiltration is observed by injection in the immature or regressed recipient testis than in the normal adult testis, presumably because intratubular fluid pressure is not high and allows more fluid to enter the seminiferous tubules in the retrograde direction in the immature or regressed testis [71]. Furthermore, the efficiency of the infusion is greatly increased by reducing the number of endogenous germ cells in the recipient testes [72], because these testes are thought to be reduced the competition to access toward the niche in the basal compartment of seminiferous tubules between the transplanted spermatogonia and endogenous germ cells [6,59,73,74]. However, the mechanism underlying migration of germ cells from the luminal compartment through the blood-testis barrier to the base of the seminiferous epithelium remains unknown.

Methods to degenerate endogenous spermatogenic cells: Germ cell transplantation is seemingly more successful if endogenous spermatogenesis is reduced or stopped in the recipient testis. Several
pretreatment methods can be used to prepare the recipient testis, such as busulfan treatment [6,52,75], irradiation [76,77], cold ischemia [78], hyperthermic treatment [79,80], or heat shock [81]. Both tests from strains that inherently lack spermatogenesis or immature can also serve as useful recipients.

Although germ cell ablation by treatment with busulfan has no known effects on intratesticular levels of testosterone [67,82,83], it affects the gene expression of Sertoli cells [84]. Furthermore, the effect of irradiation on Sertoli cells seems to depend on the age of the animal. Irradiation before terminal differentiation of Sertoli cells in the rhesus monkey and rat causes loss of Sertoli cells [85,86]. However, it has been reported that a fractionated dose of 1.5–12 Gy of X-rays at 24-hour intervals stops endogenous spermatogenesis and adequately removes all spermatogenic cells, but does not have an apparently harmful effect on adult Sertoli cells [64,87]. Because Sertoli cells play an important role in establishing an appropriate niche for spermatogenic cells to proliferate or differentiate, the treatment used for ablation of the spermatogenic cells should be carefully selected to reduce the effect on Sertoli cells in the recipient testis.

Recipient age: In contrast to the donor age effect for cell transplantation described previously, the microenvironment is better in the immature mouse testis than in adult mouse testis for allowing a wide colonization area, regardless of whether the donor cells are from an adult or immature mouse [62]. Shinohara et al. [62] suggested that the immature mouse recipient testis lacks the Sertoli cell junctions, resulting in easy stem cell migration into the basement membrane of the seminiferous tubules.

Another problem regarding quality of sperm: Although live mouse offspring can be produced using sperm derived from spermatogonial transplantation, sperm safety should be assessed carefully. Litter sizes after mating are smaller for mice after transplantation than for normal fertile mice [88,89]. Goossens E et al. reported that the sperm quality with respect to aspects such as concentration, motility, and hyperactivity was lower in sperm produced by germ cell transplantation than in control sperm and that this resulted in a reduced fertilization rate after in vitro fertilization (IVF) [88]. Mammalian spermatogenic cells differentiated into spermatocytes in the testis but not into mature sperms [90,91]. Because sperm maturation occurs in the epididymis and not in the testis [90,91], there is a possibility that spermatocytes produced by transplantation into the testis also do not mature, thereby resulting in the lower fertilization rate in IVF. Furthermore, because sperm quality may be related to the developmental process of spermatogenesis, the limitations of normal spermatogenesis by the germ cell transplantation method must be taken into consideration.

Factors Affecting Spermatogenesis Using In vitro Systems

Two in vitro spermatogenesis models have been shown to provide complete spermatogenesis [4,8-10]: tissue culture with or without germ cell transplantation and 3D testicular cell culture. The greatest advantage of these methods is that they can induce complete spermatogenesis from SSCs under in vitro conditions, possibly allowing easier analysis of factors that affect spermatogenesis than in vivo models would. However, these methods are more complicated than single cell culture or 2 dimensional (2D) cell culture methods because all these systems require a complex of testicular cells, such as tissue or aggregated testicular cells. Thus, it is still difficult to analyze the cell-to-cell interaction during spermatogenesis in real time even when using these methods. In addition, these methods are currently only applicable to mice.

Tissue culture with or without germ cell transplantation

Compared to other cell culture methods, tissue culture has an advantage with regard to the maintenance of the microenvironment caused by morphological structure. Sato et al. [8] showed in vitro production of functional mouse sperm by using the gas-liquid interface neonatal tissue culture method. Furthermore, they showed that cryopreserved neonatal mouse testicular tissue fragments were capable of spermatogenesis under this in vitro condition. Compared to the traditional tissue culture methods, this unique culture method uses an agarose gel half-soaked in medium for the gas-liquid interface system, and knockout serum (KSR) or lipid-rich bovine serum albumin (AlbuMAX) is used instead of fetal bovine serum (FBS) for culture medium supplementation. The possibility of achieving spermatogenesis by using adult testicular tissue or by using neonatal testicular tissue from another mammalian species by this culture method is presently unknown. Increased knowledge regarding these aspects will help contribute to elucidating the molecular mechanisms underlying spermatogenesis and the development of diagnostic and therapeutic techniques because it will be easier to manage the environmental conditions of the tissues in these systems than in in vivo systems.

Compared to the tissue culture technique, the combined tissue culture and germ cell transplantation technique has an advantage in that it allows analysis of cell-to-cell interactions in cultured testicular tissue although there is a limitation with respect to observing the interactions directly during culture. Sato et al. recently reported that SSCs and cultured SSCs from cryptorchid adult testes or immature testes can be used to produce fertile spermatids and sperm in vitro by using a combination of the cell transplantation technique and organ culture method [9]. For the recipient testes, they used busulfan-treated wild-type or W/Wv adult mice testes with depleted endogenous germ cells. In the explanted recipient testis tissues, SSCs were found to differentiate occasionally into spermatids and further into sperm in some cases for the endogenous germ cell-depleted mouse testes, whereas SSCs differentiated into spermatids in some cases but not into sperm in the adult wild-type testes [9]. Endogenous germ cell depletion appears to be favorable for donor germ cell colonization in vitro as well as in vivo. Successful spermatogenesis in cross-species germ cell transplantation in vivo has been shown only between rats and mice, and it may be difficult to observe spermatogenesis in other mammals under these combined culture conditions. However, it is possible to establish a tissue culture system with allogenic germ cell transplantation using other animals for recipient tissues because it might be easier to prepare both tissue fragments and germ cells from the same species or congeners for in vitro systems than to prepare donor animals for in vivo systems. Many aspects of the cell transplantation technique can be used to develop this method for practical use.

3D germ cell culture system

The 3D germ cell culture system has an advantage over tissue culture systems with respect to the ability to handle individual cells separately. Lee et al. [92,93] showed post-meiotic differentiation of spermatocytes by 3D culture of rat testicular cells and human testicular cells in a collagen matrix gel. Recently, transmeiotic differentiation of murine male germ cells has been reported in a soft-agar culture system (SACS) [4,10]; this system was initially used for hematopoietic cell culture [94] and has been adopted for testicular cell culture by Stukenberget al. [95]. The SACS or methylcellulose culture system (MCS) [10,95] can
provide a microenvironment that resembles the 3D in situ organization of the seminiferous epithelium. However, because of the characteristics of the matrix, SACS and MCS require a thick layer, in contrast to conventional cell cultures, which require a thin layer of outer cellular matrix. Furthermore, the cultured cells in the matrix aggregate as in 3D formation [10,95]. These features of the culture conditions cause difficulties in observing cell-to-cell interactions directly during culture.

However, SACS has an advantage over the tissue culture system for analyzing the cellular interaction between germ cells and somatic cells because it is possible to create more optimal experimental conditions using germ cells with or without direct contact with the somatic cells that exist in a different zone of the soft agar layers [10]. Stuckenborg et al. [10] showed that adding somatic cells to the solid lower phase of the soft agar layer resulted in more extensive colony formation and improved spermatogenic differentiation of the germ cell fraction in the upper gel phase than single germ cell culture in the soft agar layer. Furthermore, they found that the cells formed dense aggregates in the matrices and finally differentiated into spermatozoa when all cell types from the immature mouse testis, including Leydig cells, Sertoli cells, myoid cells, and germ cells, in the SACS were cultured with gonadotropins. Thus, the presence of somatic cells is necessary for efficient proliferation of germ cells in vitro by using SACS and the adequate ratio of each kind of somatic cells for combination culture with germ cells may improve the results of spermatogenesis.

The expansion and maintenance of meiotic germ cells is hormone dependent, while meiotic and post-meiotic development appears to occur independently of hormones under the conditions used in the SACS [95]. A very recent study shows that the differentiated spermatogonia produced by this culture system without hormone supplements but with fetal calf serum from all testicular cells of immature mouse are morphologically normal, although their fertility has not been confirmed yet [4]. The sperm can be detected only after fixation because of either the difficulties in microscopically detecting sperms in the thick agar layer or the limited number of sperm. Although 3D cell culture models need to be improved with regard to low sperm production, several knowledge of isolated testicular cell grafting and the other in vitro systems can be used to develop this method for practical use.

Conclusion and Perspective

All the methods that have been described have many advantages with respect to studying spermatogenesis and preserving animal fertility. However, all these methods have some limitations. For example, xenografting methods can be used for inducing spermatogenesis in many animals but it is difficult to observe spermatogenesis in real time using these methods minutely, however, a trial involving rats expressing GFP is currently underway (SD-Tg[CG-EGFP]/CZ-004Ob rat) [23]. A disadvantage of the germ cell transplantation method is its inconvenience for use with cross species transplantation, with the exception of transplantation between the rat and mouse. However, given the ability of the germ cell transplantation method to increase the spermatogonial cells in any species, this method has a great advantage with respect to studying proliferation of SSCs and preservation of the SSCs from immature animals or wild animals facing extinction. Because both grafting and germ cell transplantation use donor animals, it is necessary to be aware that the environmental conditions of these systems are still unclear. Unlike in vitro models, in which tissue culture is performed with or without germ cell transfer, in vitro models have recently been developed using mice. More information about these models is required and may be obtained by using different animal species under different conditions. Furthermore, 3D cell culture models may pave the way for studying the interaction of germ cells and somatic cells in the testes after issues such as the production of a small number of spermatozoa and unclear vision for real-time analysis of the cells have been resolved. In addition, because there are potential risks of transferring cancer cells or viruses when using human tissues or cells in these methods, many safety- and ethics-related problems should be resolved before these systems are used for clinical applications.

In conclusion, in vivo and in vitro spermatogenesis models have many advantages and disadvantages, and suitable models must be chosen depending on the requirements, after independently considering these aspects.

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