Journal of Veterinary Science & Technology

Sato et al., J Veterinar Sci Technolo 2012, 3:1
DOI: 10.4172/2157-7579.1000115

Review Article Open Access

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 vivo* 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 spermatozoa 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 spermatozoa 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 spermatozoa 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 immunodeficient mouse hosts results in germ cell differentiation and production of sperm from mammalian species like 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 spermatozoa 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-yr-old rhesus monkeys) donors have been reported to survive better than grafted tissue from other older mature 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

Received February 15, 2012; **Accepted** May 24, 2012; **Published** May 30, 2012

Citation: Sato Y, Taniguchi M, Otoi T (2012) Studying Spermatogenesis by using *In vivo* and *In vitro* Models: Advantages and Disadvantages of these Models for Practical use. J Veterinar Sci Technol 3:115. doi:10.4172/2157-7579.1000115

	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) - (13)	+ (31) - (57)	- (8,9)	ND
Cross species	- (7,13,14,15,16,17,18) + (19)	+ (54,55,56,57,58)	ND	ND
Recipient location	subcutaneous- (7,13,14,15,16,17,18,19,20) (in some cases, +) (47,50)	+ (71)	ND	ND
Recipient treatment	- (38,46)	+ (85,86,53)	+(combi) (9)	ND
Recipient age	+ (39)	+ (62)	ND	ND
Others	acceleration of spermatogenesis (7,16,20)	decrease in litter size (88,89)	only mouse data (8,9) small number of sperm (combi) (68)	only mouse data (10) small number of sperm (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.

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 point of transplantation may be responsible. The grafting tissue is subjected to ischemic conditions from the beginning of the procedure until angiogenesisis 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 [13]. 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 testicular tissue cryopreservation with the 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 [13] 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

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 showed spermatogenesis, although the graft size was smaller than that in male recipient mice with intact testis [38]. The lack of differences between intact and gonadectomized recipient mice for spermatogenesis in the testicular tissue xenografts implies that a transient increase in the serum level of gonadotropins may not be required for the initiation of spermatogenesis in xenografts. Further information is required regarding the hormonal milieu for spermatogenesis in xenografts.

Recipient age and species: Ehmcke et al. reported that grafting of neonatal hamster tissue into young and aged recipient immunodeficient mice caused increased spermatogenic activity and decreased fibrosis to a greater extent in aged recipients than in young ones [39]. They speculated that immunosenesence in the aged recipients may have affected the results because immunodeficient nude mice possess most other immune systems without mature T cells [40]. Because young nude mice possess T cell precursors and produce mature T cells over time, aged nude mice might show lower immunodeficiency than young nude mice. In terms of spermatogenic development, porcine testicular grafts from severe combined immunodeficient (SCID) mice, which lack both T and B lymphocytes, tend to display higher levels of development than those from nude mice [38,41]. However, equine testicular grafts do not show any difference between the host strains [18]. Furthermore, NOG mice (NOD/Shi-SCID, IL-2Rγc^{null}), which are more severely immunocompromised than nude and SCID mice [42], do not show the most successful development of porcine spermatogenesis among 3host 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 spermatozoa, this method used for spermatogonia 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 testes 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 spermatozoa 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 spermatozoa 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 tissue, 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 tissue 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 spermatozoa 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[CAG-EGFP]CZ-004Osb* 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 vivo* 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, 3Dcell 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.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science to Y.S. (21570228) and Heiwa Nakajima Foundation to T.O.

References

1. Russell LD, Ettlin RA, Hikim APS, Clegg ED (1990) Histological and Histopathological evaluation of the Testis. Cashe River Press.
2. Jamsai D, O'Bryan MK (2011) Mouse models in male fertility research. Asian J Androl 13:139-151.
3. Rossi P, Sette C, Dolci S, Geremia R (2000) Role of c-kit in mammalian spermatogenesis. J Endocrinol Invest 23: 609-615.
4. Abu Elhija M, Lunenfeld E, Schlatt S, Huleihel M (2011) Differentiation of murine male germ cells to spermatozoa in a soft agar culture system. Asian J Androl 14: 285-293.
5. Brinster RL, Avarbock MR (1994) Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci USA 91:11303-11307.
6. Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation.Proc Natl Acad Sci U S A 91:11298-11302.
7. Honaramooz A, Snedaker A, Boiani M, Schöler H, Dobrinski I, et al. (2002) Sperm from neonatal mammalian testes grafted in mice. Nature 418: 778-781.
8. Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, et al. (2011) *In vitro* production of functional sperm in cultured neonatal mouse testes. Nature 471: 504-507.
9. Sato T, Katagiri K, Yokonishi T, Kubota Y, Inoue K, et al. (2011) *In vitro* production of fertile sperm from murine spermatogonial stem cell lines. Nat Commun 2: 472.
10. Stukenborg JB, Schlatt S, Simoni M, Yeung CH, Elhija MA, et al. (2009) New horizons for *in vitro* spermatogenesis? An update on novel three-dimensional culture systems as tools for meiotic and post-meiotic differentiation of testicular germ cells. Mol Hum Reprod 15: 521-529.
11. Goel S, Reddy N, Mahla RS, Suman SK, Pawar RM (2011) Spermatogonial stem cells in the testis of an endangered bovid: Indian black buck (Antilope cervicapra L).Anim Reprod Sci 126: 251-257.
12. Wyns C, Curaba M, Vanabelle B, Van Langendonckt A, Donnez J (2010) Options for fertility preservation in prepubertal boys. Hum Reprod Update 16: 312-328.
13. Schlatt S, Kim SS, Gosden R (2002) Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and heterotopic grafting to castrated hosts. Reproduction 124: 339-346.
14. Shinohara T, Inoue K, Ogonuki N, Kanatsu-Shinohara M, Miki H, et al. (2002) Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro microinsemination. Hum Reprod 17: 3039-3045.
15. Oatley JM, de Avila DM, Reeves JJ, McLean DJ (2004) Spermatogenesis and germ cell transgene expression in xenografted bovine testicular tissue. Biol Reprod 71: 494-501.
16. Honaramooz A, Li MW, Penedo MC, Meyers S, Dobrinski I (2004) Accelerated

maturation of primate testis by xenografting into mice. Biol Reprod 70:1500-1503.

17. Snedaker AK, Honaramooz A, Dobrinski I (2004) A game of cat and mouse: xenografting of testis tissue from domestic kittens results in complete cat spermatogenesis in a mouse host. J Androl 25: 926-930.

18. Rathi R, Honaramooz A, Zeng W, Turner R, Dobrinski I (2006) Germ cell development in equine testis tissue xenografted into mouse. Reproduction 131:1091-1098.

19. Wistuba J, Luetjens CM, Wesselmann R, Nieschlag E, Simoni M, et al. (2006) Meiosis in autologous ectopic transplants of immature testicular tissue grafted to Callithrix jacchus. Biol Reprod 74: 706-713.

20. Sato Y, Nozawa S, Yoshiike M, Arai M, Sasaki C, et al. (2010) Xenografting of testicular tissue from an infant human donor results in accelerated testicular maturation. Hum Reprod 25:1113-1122.

21. Nakai M, Kaneko H, Somfai T, Maedomari N, Ozawa M, et al. (2010) Production of viable piglets for the first time using sperm derived from ectopic testicular xenografts. Reproduction 139: 331-335.

22. Arregui L, Rathi R, Zeng W, Honaramooz A, Gomendio M, et al. (2008) Xenografting of adult mammalian testis tissue. Anim Reprod Sci 106: 65-76.

23. Schlatt S, Westernströer B, Gassei K, Ehmcke J (2010) Donor-host involvement in immature rat testis xenografting into nude mouse hosts. Biol Reprod 82: 888-895.

24. Bajpai M, Gupta G, Setty BS (1998) Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat. Eur J Endocrinol 138: 322-327.

25. Schmidt JA, de Avila JM, McLean DJ (2006) Grafting period and donor age affect the potential for spermatogenesis in bovine ectopic testis xenografts. Biol Reprod 75:160-166.

26. Schmidt JA, de Avila JM, McLean DJ (2006) Effect of vascular endothelial growth factor and testis tissue culture on spermatogenesis in bovine ectopic testis tissue xenografts. Biol Reprod 75:167-175.

27. Li J, Savolainen H, Tan F, Zheng S (2010) Orthotopic testicular transplantation in mice. Reproduction 139: 447-452.

28. Schlatt S, Honaramooz A, Ehmcke J, Goebell PJ, Rübben H, et al. (2006) Limited survival of adult human testicular tissue as ectopic xenograft. Hum Reprod 21: 384-389.

29. Turner RM, Rathi R, Honaramooz A, Zeng W, Dobrinski I (2010) Xenografting restores spermatogenesis to cryotprchid testicular tissue but does not rescue the phenotype of idiopathic testicular degeneration in the forse (Equus caballus). Reprod Feritil Dev 22: 673-683.

30. Arregui L, Rathi R, Modelski M, Zeng W, Roldan ER, et al. (2012) Suppression of spermatogenesis before grafting increases survival and supports resurgence of spermatogenesis in adult mouse testis. Fertil Steril.

31. Kanatsu-Shinohara M, Ogonuki N, Inoue K, Ogura A, Toyokuni S, et al. (2003) Restoration of fertility in infertile mice by transplantation of cryopreserved male germline stem cells. Hum Reprod 18: 2660-2667.

32. Wyns C, Curaba M, Martinez-Madrid B, Van Langendonckt A, François-Xavier W, et al. (2007) Spermatogonial survival after cryopreservation and short-term orthotopic immature human cryptorchid testicular tissue grafting to immunodeficient mice. Hum Reprod 22:1603-1611.

33. Zeng W, Snedaker AK, Megee S, Rathi R, Chen F, et al. (2009) Preservation and transplantation of porcine testis tissue. Reprod Fertil Dev 21: 489-497.

34. Jahnukainen K, Ehmcke J, Hergenrother SD, Schlatt S (2007) Effect of cold storage and cryopreservation of immature non-human primate testicular tissue on spermatogonial stem cell potential in xenografts. Hum Reprod 22: 1060-1067.

35. Christl HW (1990) The lamina propria of vertebrate seminiferous tubules: a comparative light and electron microscopic investigation. Andrologia 22: 85-94.

36. Setchell BP (1978) The Mammalian Testis. Cornell University Press; Ithaca, NY.

37. Schlatt S, Honaramooz A, Boiani M, Scholer HR, Dobrinski I (2003) Progeny from sperm obtained after ectopic grafting of neonatal mouse testes. Biol Reprod 68: 2331-2335.

38. Abbasi S, Honaramooz A (2011) Effects of recipient mouse strain, sex and gonadal status on the outcome of testis tissue xenografting. Reprod Fertil Dev 22:1279-1286.

39. Ehmcke J, Gassei K, Schlatt S (2008) Ectopic testicular xenografts from newborn hamsters (Phodopus sungorus) show better spermatogenic activity in aged compared with young recipients.J Exp Zool A Ecol Genet Physiol 309: 278-287.

40. Pelleitier M, Montplaisir S (1975) The nude mouse: a model of deficient T-cell function. Methods Achiev Exp Pathol 7: 149-166.

41. Watanabe T, Hayashi H, Kita K, Kubota Y, Ogawa T (2009) Ectopic porcine spermatogenesis in murine subcutis: tissue grafting versus cell-injection methods. Asian J Androl 11: 317-323.

42. Ito M, Hiramatsu H, Kobayashi K, Suzue K, KawahataM, et al. (2002) NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. 100: 3175–3182.

43. Oatley JM, Reeves JJ, McLean DJ (2005) Establishment of spermatogenesis in neonatal bovine testicular tissue following ectopic xenografting varies with donor age. Biol Reprod 72: 358-364.

44. Honaramooz A, Megee SO, Rathi R, Dobrinski I (2007) Building a testis: formation of functional testis tissue after transplantation of isolated porcine (Sus scrofa) testis cells. Biol Reprod 76: 43-47.

45. Kita K, Watanabe T, Ohsaka K, Hayashi H, Kubota Y, et al. (2007) Production of functional spermatids from mouse germline stem cells in ectopically reconstituted seminiferous tubules. Biol Reprod 76: 211-217.

46. Wistuba J, Mundry M, Luetjens CM, Schlatt S (2004) Cografting of hamster (Phodopus sungorus) and marmoset (Callithrix jacchus) testicular tissues into nude mice does not overcome blockade of early spermatogenic differentiation in primate grafts. Biol Reprod71: 2087-2091.

47. Luetjens CM, Stukenborg JB, Nieschlag E, Simoni M, Wistuba J (2008) Complete spermatogenesis in orthotopic but not in ectopic transplants of autologously grafted marmoset testicular tissue. Endocrinology 149:1736-1747.

48. Gromoll J, Wistuba J, Terwort N, Godmann M, Müller T, et al. (2003) A new subclass of the luteinizing hormone/chorionic gonadotropin receptor lacking exon 10 messenger RNA in the New World monkey (Platyrrhini) lineage. Biol Reprod 69: 75-80.

49. Müller T, Gromoll J, Simula AP, Norman R, Sandhowe-Klaverkamp R, et al. (2004) The carboxyterminal peptide of chorionic gonadotropin facilitates activation of the marmoset LH receptor. Exp Clin Endocrinol Diabetes 112: 574-579.

50. Luetjens CM, Weinbauer GF, Wistuba J (2005) Primate spermatogenesis: new insights into comparative testicular organisation, spermatogenic efficiency and endocrine control. Biol Rev Camb Philos Soc 80: 475-488.

51. Clouthier DE, Avarbock MR, Maika SD, Hammer RE, Brinster RL (1996) Rat spermatogenesis in mouse testis. Nature381:418–421.

52. Ogawa T, Dobrinski I, Brinster RL (1999) Recipient preparation is critical for spermatogonial transplantation in the rat. Tissue Cell 31: 461–472.

53. Young GP, Goldstein M, Phillips DM, Sundaram K, Gunsalus GL, et al. (1988) Sertoli cell-only syndrome produced by cold testicular ischemia. Endocrinology 122:1074-1082.

54. Dobrinski I, Avarbock MR, Brinster RL (1999) Transplantation of germ cells from rabbits and dogs into mouse testes. Biol Reprod 61:1331-1339.

55. Dobrinski I, Avarbock MR, Brinster RL (2000) Germ cell transplantation from large domestic animals into mouse testes. Mol Reprod Dev 57: 270-279.

56. Nagano M, McCarrey JR, Brinster RL (2001) Primate spermatogonial stem cells colonize mouse testes. Biol Reprod 64:1409-1416.

57. Nagano M, Patrizio P, Brinster RL (2002) Long-term survival of human spermatogonial stem cells in mouse testes. Fertil Steril 78: 1225-1233.

58. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL (1999) Xenogeneic spermatogenesis following transplantation of hamster germ cells to mouse testes. Biol Reprod 60: 515-521.

59. Honaramooz A, Behboodi E, Blash S, Megee SO, Dobrinski I (2003) Germ cell transplantation in goats. Mol Reprod Dev 64: 422-428.

60. Rockett JC, Mapp FL, Garges JB, Luft JC, Mori C, et al. (2001) Effects of

hyperthermia on spermatogenesis, apoptosis, gene expression, and fertility in adult male mice. Biol Reprod 65: 229-239.

61. Sato T, Aiyama Y, Ishii-Inagaki M, Hara K, Tsunekawa N, et al. (2011) Cyclical and Patch-Like GDNF Distribution along the Basal Surface of Sertoli Cells in Mouse and Hamster Testes. PLoS One6: e28367.

62. Shinohara T, Orwig KE, Avarbock MR, Brinster RL (2001) Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc Natl Acad Sci U S A 98: 6186-6191.

63. Avarbock MR, Brinster CJ, Brinster RL (1996) Reconstitution of spermatogenesis from frozen spermatogonial stem cells. Nat Med 2: 693-696.

64. Izadyar F, Matthijs-Rijsenbilt JJ, den Ouden K, Creemers LB, Woelders H, et al. (2002) Development of a cryopreservation protocol for type A spermatogonia. J Androl 23: 537-545.

65. Sofikitis N, Kaponis A, Mio Y, Makredimas D, Giannakis D, (2003) Germ cell transplantation: a review and progress report on ICSI from spermatozoa generated in xenogeneic testes. Hum Reprod Update 9: 291-307.

66. de Rooji DG, Russell LD (2000) All you wanted to know about spermatogonia but were afraid to ask. J Androl 21: 776-798.

67. Morris ID, Bardin CW, Musto NA, Thau RB, Gunsalus GL (1987) Evidence suggesting that germ cells influence the bidirectional secretion of androgen binding protein by the seminiferous epithelium demonstrated by selective impairment of spermatogenesis with busulphan. Int J Androl 10: 691–700.

68. Ogawa T, Aréchaga JM, Avarbock MR, Brinster RL (1997)Transplantation of testis germinal cells into mouse seminiferous tubules. Int J Dev Biol 41: 111-122.

69. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL (2000) Transplantation of male germ line stem cells restores fertility in infertile mice. Nat Med 6: 29-34.

70. Russel LD, Nagano M, Brinster RL (1998) Spermatogonial transplantation. In Stefanini et al. (eds), Testicular function: From gene expression to genetic manipulation. Springer, Berlin, pp.41-57.

71. Schlatt S, Rosiepen G, Weinbauer GF, Rolf C, Brook PF, et al. (1999) Germ celltransfer into rat, bovine, monkey and human testes. Hum Reprod 14:144-150.

72. Honaramooz A, Behboodi E, Hausler CL, Blash S, Ayres S, et al. (2005) Depletion of endogenous germ cells in male pigs and goats in preparation for germ cell transplantation. J Androl 26: 698-705.

73. Honaramooz A, Behboodi E, Megee SO, Overton SA, Galantino-Homer H, et al. (2003) Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. Biol Reprod 69:1260-1264.

74. Meistrich ML, van Beek MEAB (1993) Spermatogonial stem cells. In Desjardins C and Ewing LL (eds). Cell and Molecular Biology of the Testis. Oxford University Press, New York, USA, pp. 266-195.

75. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL (1998) Leuprolide, a gonadotropin-releasing hormone agonist, enhances colonization after spermatogonial transplantation into mouse testes. Tissue Cell 30: 583-538.

76. Shuttlesworth GA, de Rooij DG, Huhtaniemi I, Reissmann T, Russell LD, et al. (2000) Enhancement of A spermatogonial proliferation and differentiation in irradiated rats by gonadotropin-releasing hormone antagonist administration. Endocrinology 141: 37-49.

77. van den Aardweg GJ, de Ruiter-Bootsma AL, Kramer MF, Davids JA (1983) Growth and differentiation of spermatogenetic colonies in the mouse testis after irradiation with fission neutrons. Radiat Res 94: 447-463.

78. Young GP, Goldstein M, Phillips DM, Sundaram K, Gunsalus GL, et al. (1988) Sertoli cell-only syndrome produced by cold testicular ischemia. Endocrinology 122:1074-1082.

79. McLean DJ, Russell LD, Griswold MD (2002) Biological activity and enrichment of spermatogonial stem cells in vitamin A-deficient and hyperthermia-exposed testes from mice based on colonization following germ cell transplantation. Biol Reprod 66:1374-1379.

80. Rockett JC, Mapp FL, Garges JB, Luft JC, Mori C, et al. (2001) Effects of hyperthermia on spermatogenesis, apoptosis, gene expression, and fertility in adult male mice. Biol Reprod 65: 229-239.

81. Ma W, An L, Wu Z, Wang X, Guo M, et al. (2011) Efficient and safe recipient preparation for transplantation of mouse spermatogonial stem cells: pretreating testes with heat shock. Biol Reprod 85: 670-677.

82. De Franca LR, Bartke A, Borg KE, Cecim M, Fadden CT, et al. (1994) Sertoli cells in testes containing or lacking germ cells: a comparative study of paracrine effects using the W (c-kit) gene mutant mouse model. Anat Rec 240: 225–232.

83. Gomes WR, Hall RW, Jain SK, Boots LR (1973) Serum gonadotropin and testosterone levels during loss and recovery of spermatogenesis in rats. Endocrinology 93: 800-809.

84. O'Shaughnessy PJ, Hu L, Baker PJ (2008) Effect of germ cell depletion on levels of specific mRNA transcripts in mouse Sertoli cells and Leydig cells. Reproduction 135: 839-850.

85. de Rooij DG, van de Kant HJ, Dol R, Wagemaker G, van Buul PP, et al. (2002) Long-term effects of irradiation before adulthood on reproductive function in the male rhesus monkey. Biol Reprod 66: 486-494.

86. Erickson BH, Blend MJ (1976) Response of the Sertoli cell and stem germ cell to 60Co gamma-radiation (dose and dose rate) in testes of immature rats. Biol Reprod 14:641-650.

87. Creemers LB, Meng X, den Ouden K, van Pelt AM, Izadyar F, et al. (2002) Transplantation of germ cells from glial cell line-derived neurotrophic factor-overexpressing mice to host testes depleted of endogenous spermatogenesis by fractionated irradiation. Biol Reprod 66:1579-1584.

88. Goossens E, De Block G, Tournaye H (2008) Computer-assisted motility analysis of spermatozoa obtained after spermatogonial stem cell transplantation in the mouse.Fertil Steril 90: 1411-1416.

89. Goossens E, Frederickx V, de Block G, van Steirteghem A, Tournaye H (2006) Evaluation of *in vivo* conception after testicular stem cell transplantation in a mouse model shows altered post-implantation development. Hum Reprod 21: 2057-2060.

90. Robaire B, Hermo L (1988) Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. In: Knobil E, Neil JD (eds). The Physiology of Reproduction. Raven Press, New York, pp. 999–1079.

91. Turner TT (1991) Spermatozoa are exposed to a complex microenvironment as they traverse the epididymis. Ann N Y Acad Sci 637: 364-383.

92. Lee JH, Gye MC, Choi KW, Hong JY, Lee YB, et al. (2007) *In vitro* differentiation of germ cells from nonobstructive azoospermic patients using three-dimensional culture in a collagen gel matrix. Fertil Steril 87: 824-833.

93. Lee JH, Kim HJ, Kim H, Lee SJ, Gye MC (2006) *In vitro* spermatogenesis by three-dimensional culture of rat testicular cells in collagen gel matrix. Biomaterials 27: 2845-2853.

94. Parent-Massin D (2001) Relevance of clonogenic assays in hematotoxicology. Cell Biol Toxicol1 7: 87-94.

95. Stukenborg JB, Wistuba J, Luetjens CM, Elhija MA, Huleihel M, (2008) Coculture of spermatogonia with somatic cells in a novel three-dimensional soft-agar-culture-system. J Androl 29: 312-329.