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Review of injection techniques for spermatogonial stem cell transplantation

Murat Gul [1,2,*], Simone Hildorf [3], Lihua Dong [1], Jorgen Thorup [3], Eva R. Hoffmann [4], Christian Fuglesang S Jensen [5], Jens Sønksen [5,6], Dina Cortes [6,7], Jens Fedder [8,9], Claus Yding Andersen [1,6,†], and Ellen Goossens [10,†]

[1]Laboratory of Reproductive Biology, Copenhagen University Hospital Rigshospitalet, 2100 Copenhagen, Denmark [2]Department of Urology, Selcuk University School of Medicine, 42250 Konya, Turkey [3]Department of Pediatric Surgery, Copenhagen University Hospital Rigshospitalet, 2100 Copenhagen, Denmark [4]DNRF Center for Chromosome Stability, Department of Molecular and Cellular Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, 2200 Copenhagen, Denmark [5]Department of Urology, Herlev and Gentofte University Hospital, 2930 Herlev, Denmark [6]Faculty of Health and Medical Sciences, University of Copenhagen, 2200 Copenhagen, Denmark [7]Department of Pediatrics, Copenhagen University Hospital Hvidovre, 2650 Hvidovre, Denmark [8]Centre of Andrology & Fertility Clinic, Department D, Odense University Hospital, 5000 Odense, Denmark [9]Research Unit of Human Reproduction, Institute of Clinical Research, University of Southern Denmark, 5230 Odense, Denmark [10]Biology of the Testis, Research Laboratory for Reproduction, Genetics and Regenerative Medicine, Vrije Universiteit Brussel (VUB), 1090 Brussels, Belgium

*Correspondence address. Laboratory of Reproductive Biology Rigshospitalet, Copenhagen University, Hospital Rigshospitalet, Section 5701, Henrik Harpestrengsvej 4, 2100 Copenhagen, Denmark. Tel: (+45) 5013 0232; E-mail: drgulacademics@gmail.com
https://orcid.org/0000-0002-6657-6227

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[†]Claus Yding Andersen and Ellen Goossens share the senior authorship

BACKGROUND: Although the prognosis of childhood cancer survivors has increased dramatically during recent years, chemotherapy and radiation treatments for cancer and other conditions may lead to permanent infertility in prepubertal boys. Recent developments have shown that spermatogonial stem cell (SSC) transplantation may be a hope for restoring fertility in adult survivors of childhood cancers. For this reason, several centres around the world are collecting and cryopreserving testicular tissue or cells anticipating that, in the near future, some patients will return for SSC transplantation. This review summarizes the current knowledge and utility of SSC transplantation techniques.

OBJECTIVE AND RATIONALE: The aim of this narrative review is to provide an overview of the currently used experimental injection techniques for SSC transplantation in animal and human testes. This is crucial in understanding and determining the role of the different techniques necessary for successful transplantation.

SEARCH METHODS: A comprehensive review of peer-reviewed publications on this topic was performed using the PubMed and Google Scholar databases. The search was limited to English language work and studies between 1994 (from the first study on SSC transplantation) and April 2019. Key search terms included mouse, rat, boar, ram, dog, sheep, goat, cattle, monkey, human, cadaver, testes, SSC transplantation, injection and technique.

OUTCOMES: This review provides an extensive clinical overview of the current research in the field of human SSC transplantation. Rete testis injection with ultrasonography guidance currently seems the most promising injection technique thus far; however, the ability to draw clear conclusions is limited due to long ischemia time of cadaver testis, the relatively decreased volume of the testis, the diminishing size of seminiferous tubules, a lack of intratesticular pressure and leakage into the interstitium during the injection on human cadaver testis. Current evidence does not support improved outcomes from multiple infusions through the rete testes. Overall, further optimization is required to increase the efficiency and safety of the infusion method.

WIDER IMPLICATIONS: Identifying a favourable injection method for SSC transplantation will provide insight into the mechanisms of successful assisted human reproduction. Future research could focus on reducing leakage and establishing the optimal infusion cell concentrations and pressure.

Key words: efferent duct / male infertility / injection / rete testis / seminiferous tubule / stem cell transplantation / fertility restoration

Introduction

Spermatogonial stem cells (SSCs) are undifferentiated germ cells that are responsible for spermatogenesis throughout adulthood (de Rooij 2017). This is achieved through continuous self-renewal and initiation of a differentiation programme that produces mature spermatozoa (Ryu *et al*., 2006; Kanatsu-Shinohara and Shinohara 2013). The exact nature of SSCs in primates is still unclear (de Rooij 2017); however, in humans, the type A_{dark} (A_d) spermatogonium is probably an adult germline stem cell (Hadziselimovic and Herzog 2001; Hutson *et al*., 2016), and the appearance of this spermatogonium has been described since 1924 (Branca 1924; Roosen-Runge and Barlow 1953). Since 1963, it was called spermatogonium A_d (Clermont 1963), while, from 1966, it was called an SSC, eventually together with A_{pale} (A_p) spermatogonia (Clermont 1966; Rowley *et al*., 1971; Clermont 1972; Schulze 1978; Paniagua *et al*., 1986; Huff *et al*., 2001; von Kopylow *et al*., 2012; Thorup *et al*., 2013; Verkauskas *et al*., 2016; Di Persio *et al*., 2017).

SSC-based therapies can serve as the basis for the fertility preservation of prepubertal boys (Goossens *et al*., 2013). Testicular tissue grafting (Fayomi *et al*., 2019), SSC transplantation (Hermann *et al*., 2012) and *in vitro* spermatogenesis (de Michele *et al*., 2018) are the three major approaches to the restoration of fertility. Of these, SSC transplantation is the only approach enabling natural conception (Onofre *et al*., 2016) (Fig. 1).

Brinster *et al*. (1994) were the first to describe SSC transplantation in mice (Brinster and Avarbock 1994; Brinster and Zimmermann 1994), and, 5 years later, it was proposed as a fertility preservation method (Bahadur and Ralph 1999). Subsequently, SSC transplantation has been successful in several rodent species (Brinster and Zimmermann 1994; Clouthier *et al*., 1996; Nagano and Brinster 1998; Bahadur and Ralph 1999; Ogawa *et al*., 1999). The confirmation of the efficacy of the SSC transplantation technique (Honaramooz *et al*., 2002; Goossens *et al*., 2003; Izadyar *et al*., 2003; Hermann *et al*., 2012) as well as the demonstration of viable, healthy offspring after the long-term cryopreservation of SSCs in mice (Wu *et al*., 2012) encouraged researchers in the field to translate this technique to humans.

Although SSC transplantation is a promising prospect for fertility restoration in humans, findings from animal studies indicate that the transplantation technique is an extremely delicate and arduous procedure that needs to be optimized to achieve higher success rates before SSC transplantation can be considered a clinical tool. In this context, a more precise knowledge of the anatomy and morphology of the testis is essential for improving the efficacy of testicular transplantation in human. To address this, we performed a literature review and present the current evidence pertaining to SSC transplantation in human testes.

Beneficiaries of SSC transplantation

The cryopreservation of immature testicular tissue or cells is the only fertility preservation option for a large proportion of infant and prepubertal boys who are facing gonadotoxic treatment or diagnosed with a reproductive disorder, since they do not have mature spermatozoa that can be cryopreserved (Levine 2014; Onofre *et al*., 2016). The prepubertal testis already contains A_d and A_p spermatogonia (Paniagua and Nistal 1984), which are necessary for spermatogenesis. For this reason, several fertility centres around the world already collect and cryopreserve immature testicular tissue or cells, anticipating that SSC-based therapies will be available in the future (Picton *et al*., 2015; Gassei and Orwig 2016; Valli-Pulaski *et al*., 2019). SSC transplantation, one of

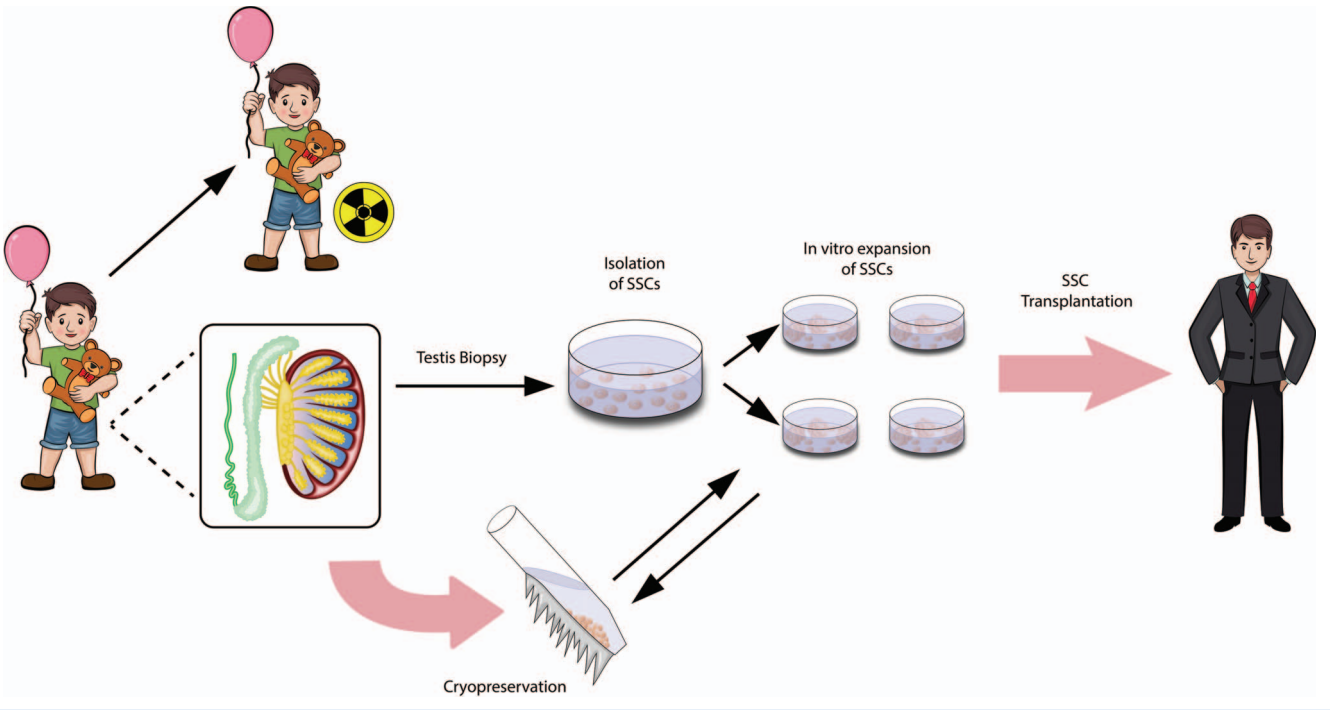


Figure 1 Clinical set-up for spermatogonial stem cell preservation in prepubertal boys at high risk of infertility. Before the exposure of the gonadotoxic treatment or expected spermatogonial stem cell (SSC) loss in the prepubertal stage, a testicular biopsy is retrieved and cryopreserved. In adulthood, when the patient is in full remission and in need of having his own offspring, cell suspensions can be thawed and propagated for autologous transplantation.

the envisioned SSC-based therapies, may restore spermatogenesis *in situ* and thereby allow patients to conceive children naturally.

Children undergoing gonadotoxic cancer therapy

With an increasing incidence of childhood cancers (Ward *et al.*, 2019a) and improved survival rates over the past half a century (Hudson 2010), there are currently 300 000 to 500 000 survivors of childhood cancer in Europe (Hjorth *et al.*, 2015). In children aged 0–14 years, acute lymphoblastic leukaemia is the most common malignancy, comprising 18–34% of cancers (Kaatsch 2010; Ward *et al.*, 2019b), while in adolescents between the ages of 15 and 19 years, Hodgkin's lymphoma is the most frequent malignancy (Ward *et al.*, 2014). Other high-incidence childhood cancers include nephroblastoma, retinoblastoma and neuroblastoma (Ward *et al.*, 2019a). Among these malignant conditions, children with leukaemia, testicular cancer or Ewing sarcoma have the highest risk of developing infertility following cancer treatment (Tournaye *et al.*, 2014).

Germ cells are highly susceptible to the toxic effects of chemotherapy during all stages of life (Meistrich 2013; Masliukaite *et al.*, 2016; Stukenborg *et al.*, 2018); however, the impact of chemotherapy on germ cells depends upon the drugs administered. Alkylating drugs, such as cyclophosphamide, cisplatin and nitrogen mustard, have the most unfavourable effect on spermatogenesis (Loren *et al.*, 2013; Tournaye *et al.*, 2014). In contrast, most patients with Hodgkin's disease who are treated doxorubicin, vinblastine, dacarbazine and bleomycin (without a high-dose alkylating agent) have a normal spermiogram and gonadotrophin levels following chemotherapy. Similarly, patients with standard-risk non-Hodgkin lymphoma or acute lymphoblastic leukaemia treated with vinca alkaloids (vincristine, vinblastine), anti-metabolites (methotrexate, mercaptopurine) and low-dose alkylating agents are at low risk of permanent infertility (Anderson *et al.*, 2015; Jahnukainen *et al.*, 2015). Although boys undergoing conventional chemotherapy are at a low risk of infertility, disease response or relapse may drive patients to sterilizing therapy.

The germinal epithelium of the testis is also very vulnerable to irradiation, and germ cell loss can be seen even at doses as low as 0.1–0.2 Gy (Howell and Shalet 2005). Patients exposed to testicular radiation >6 Gy or total body irradiation (10 to 14 Gy) are at a high risk of permanent infertility (Meistrich 2013; Anderson *et al.*, 2015; Jahnukainen *et al.*, 2015). Cumulative irradiation dose received also plays a central role in permanent sterility (Tournaye *et al.*, 2014).

According to a recently published report by a co-ordinated network of centres in which a standardized protocol was adopted for providing experimental testicular tissue cryopreservation services to patients at risk of infertility due to their medical treatments, high-risk patients were defined as patients with cyclophosphamide equivalent doses >4 g/m^2, total body irradiation (TBI), testicular radiation >2.5 Gy, cranial radiation >40 Gy and cisplatin 500 mg/m^2 (Valli-Pulaski *et al.*, 2019). Overall, patients classified at a high risk of infertility due to chemotherapy or radiotherapy could be considered potential candidates for the cryopreservation of testicular tissue, which contains SSCs (Jahnukainen *et al.*, 2015; Medrano *et al.*, 2018).

Children facing gonadotoxic therapy for non-malignant diseases

Patients with non-malignant conditions may also be subjected to gonadotoxic treatments. Sickle cell disease (SCD) is an inherited red blood cell disorder and represents a large group of patients throughout the world in which allogeneic hematopoietic stem cell transplantation requires low-dose TBI (Horan *et al*., 2005; Strouse 2016). Patients with SCD suffer from vaso-occlusion that causes organ damage and tissue hypoxia. The incidence of vaso-occlusion can be reduced by the antineoplastic agent hydroxyurea, an anti-metabolite that inhibits ribonucleotide reductase and DNA replication (DeBaun 2014). Boys undergoing this treatment are eligible for fertility preservation; however, at present, the majority will already have received treatment before a testicular tissue biopsy is offered (Jahnukainen *et al*., 2015).

The treatment regimens of other blood diseases, such as thalassemia, chronic granulomatous diseases or idiopathic medulla aplasia, may require irradiation therapy prior to hydroxyurea regimens and hemopoietic stem cell transplantation (Picton *et al*., 2015). Treatment regimens associated with allogeneic hematopoietic stem cell transplantation pose a significant risk of germ cell loss, including complete azoospermia in 72 to 85% of men and oligozoospermia in the rest (Anserini *et al*., 2002; Rovó *et al*., 2006; Kassim and Sharma 2017). Other conditions that may require allogeneic hematopoietic stem cell transplantation, such as Wiskott–Aldrich syndrome, hyper IgM syndrome, Farber disease, severe combined immune deficiency, IPEX syndrome and DOCK8 immunodeficiency syndrome, are also reported as possible indications for SSC cryopreservation (Valli-Pulaski *et al*., 2019).

Children with other benign conditions

Apart from the gonadotoxic therapies, there are several benign conditions associated with infertility.

Cryptorchidism, undescended testis, is one of the most common male genital anomalies, with an incidence of 2 to 9% in full-term boys (Cortes *et al*., 1998; Boisen *et al*., 2004). Untreated cryptorchidism is a relatively frequent cause of non-obstructive azoospermia (Barthold and Gonzalez 2003; Fedder 2011; Olesen *et al*., 2017). It is associated with a reduced number of germ cells from birth (Cortes *et al*., 1995) and germ cell loss after the first year of life (Cortes *et al*., 1995; Hadziselimovic and Herzog 2001; Kollin *et al*., 2012). Furthermore, cryptorchid boys suffer from impaired germ cell maturation (Park *et al*. 2007), Leydig cell depletion (Tasian *et al*., 2009), and testicular fibrosis (Hadziselimović *et al*., 1986). A longitudinal study on cryptorchid boys indicated that the A_d spermatogonia are a critical subtype in the establishment of spermatogenesis (Hadziselimovic and Herzog 2001). The authors compared the histological findings of the testis specimens at the time of orchidopexy (<2 years) with their counterpart semen samples from adulthood. When A_d spermatogonia were present in the biopsy at the time of orchidopexy, 94% of the men had normal sperm counts. In contrast, in the absence of A_d spermatogonia, only 8% of the men had normal sperm counts despite successful and early surgery. Around one-third of boys with cryptorchidism, especially bilateral cryptorchidism, suffered from infertility in adult life despite the presence of SSCs in the majority of the testes biopsies (Hadziselimovic and Herzog 2001). A significant association between abnormal A_d spermatogonia count at the time of orchidopexy and decreased sperm density in adulthood has also been reported (Cortes 2012, Kraft *et al*., 2012). Morevoer, a significant germ cell loss in the cryptochid testes can also be seen during the second half of the first year of life (Cortes *et al*., 1995). As a result of continuous germ cell loss, Thorup *et al*. (2018) suggested that cryopreservation may be an option for boys with cryptorchidism in cases where the treatment with adjuvant luteinizing hormone-releasing hormone agonist fails. A prerequisite for such a medical decision involves the assessment of serum hormone levels and histological findings of testicular biopsies from orchidopexy (Thorup *et al*., 2018). This would require an additional surgery for SSC preservation. However, testicular volume has also been shown as a reliable tool reflecting the germ cell number in early childhood (Kollin *et al*., 2012) facilitating the possibility of a single surgical intervention for both orchidopexy as well as SSC preservation.

Testicular cryopreservation for SSC-mediated fertility restoration relies upon the SSCs being available for propagation *in vivo* or *in vitro*. In a recent study, the successful propagation of SSC-like cells from infant boys, who underwent orchidopexy for unilateral or bilateral cryptorchidism, was demonstrated (Dong *et al*., 2019a). Therefore, boys with cryptorchidism could potentially benefit from the early cryopreservation of SSCs prior to germ cell loss.

Klinefelter syndrome (KS) is the most common chromosome disorder in males and one of the most common genetic causes of male infertility (Wikström *et al*., 2004; Gravholt *et al*., 2018; Kanakis and Nieschlag 2018). Testicular sperm extraction (TESE) combined with ICSI represents a chance for azoospermic males with KS to father children. The general success rate of finding sperm in patients with KS after TESE has been reported to be 40% (Corona *et al*., 2017). Currently, there is no fertility restoration option for adult KS patients with negative TESE (Plotton *et al*., 2015; Rohayem *et al*., 2015; Nahata *et al*., 2016). Therefore, cryopreservation of testicular biopsies from adult KS patients has been performed in several centres anticipating that cryopreserved tissues could be used to restore fertility. However, recent reports do not support continuing cryopreservation (Franik *et al*., 2016; Van Saen *et al*., 2018), since during puberty, the rising serum testosterone levels are followed by degeneration of germ cells, fibrosis of the seminiferous tubules and Leydig cell hyperplasia, all of which manifest in adulthood (Wikström *et al*., 2004; Franik *et al*., 2016; Gravholt *et al*., 2018). Critically, the numbers of germ cells in KS and control testes are similar during foetal development (Van Saen *et al*. 2018). However, germ cell numbers progressively decline from the early years of life (Mikamo *et al*., 1968; Edlow *et al*., 1969; Ratcliffe 1982; Van Saen *et al*., 2018). This suggests that testicular biopsies from the neonatal period or soon after birth may provide a window of opportunity for fertility preservation in patients with KS. Later intervention may also be feasible, but less effective. In a small study of peripubertal boys (10–14 years), 6 of 14 KS patients had biopsy specimens that contained spermatogonia A_d (Wikström *et al*., 2004). Consequently, cryopreservation of a testicular biopsy before puberty may be a possibility. As the age at diagnosis of KS is decreasing and new neonatal screening programmes are initiated, patients with KS may be beneficiaries of cryopreservation of SSCs at a very early age, prior to the onset of puberty (Berglund *et al*., 2019). Since the testes degenerate in KS individuals, fertility restoration in adulthood will depend on *in vitro* spermiogenesis.

Interspecies differences in testis architecture

Transplantation of SSC-containing testes tissue or *in vitro* propagated SSCs requires sophisticated knowledge of the human testes architecture. Critically, humans have a different testis architecture compared to rodents, domestic animals and non-human primates that are currently used as experimental model systems. Understanding the interspecies differences is therefore urgently needed to evaluate whether and how transplantation techniques from experimental animal models apply to humans (Tegelenbosch and de Rooij 1993; Almeida *et al*., 2006; Fayomi and Orwig 2018).

In this review, we focus on the anatomical and morphometric differences between the testes of adult humans and other mammals that must be considered for the successful application of SSC transplantation in human. When comparing species, we have reviewed and outlined the anatomical, stereological and morphological data of the three testicular structures, which are of interest to the transplantation sites: seminiferous tubules; rete testis; and efferent ducts. We believe that quantitative information of testicular structures, high-resolution anatomy of the testis architecture and the morphological description of the tubular network are needed to identify an efficient injection site for the future clinical application of human SSC transplantation. Figure 2 highlights such differences between the selected species.

Seminiferous tubules

In most mammals, the tunica albuginea, a fibrous capsule surrounding the testis, sends prolongations inwards, dividing the testis into closed compartments, called lobules, each of which are surrounded by a thin fibrous septum (Bonet *et al*., 2013; Dixson 2013; Treuting *et al*., 2017). Within the lobules, seminiferous tubules lie in a highly coiled and tightly packed manner. The seminiferous tubules make up the vast majority of the testis volume and contain the germinal epithelium. The degree of testis lobulation and the total volume of seminiferous tubules varies between species (Fig. 2) (Harvey and Harcourt 1984; Moller 1988; Breed 1998; Pinart *et al*., 2001; Almeida *et al*., 2006; Dixson 2013). In contrast to other species shown in Figure 2, rats do not have the similar lobular distribution of the testis but only a few fine stands of connective tissue run through the testes (Johnson *et al*., 1980; Wing and Christensen 1982; Breed 1998; Holstein *et al*., 2003; Bonet *et al*., 2013; O'Shaughnessy 2014; Nakata *et al*., 2015; Creasy and Chapin 2018; Maynard and Downes 2019).

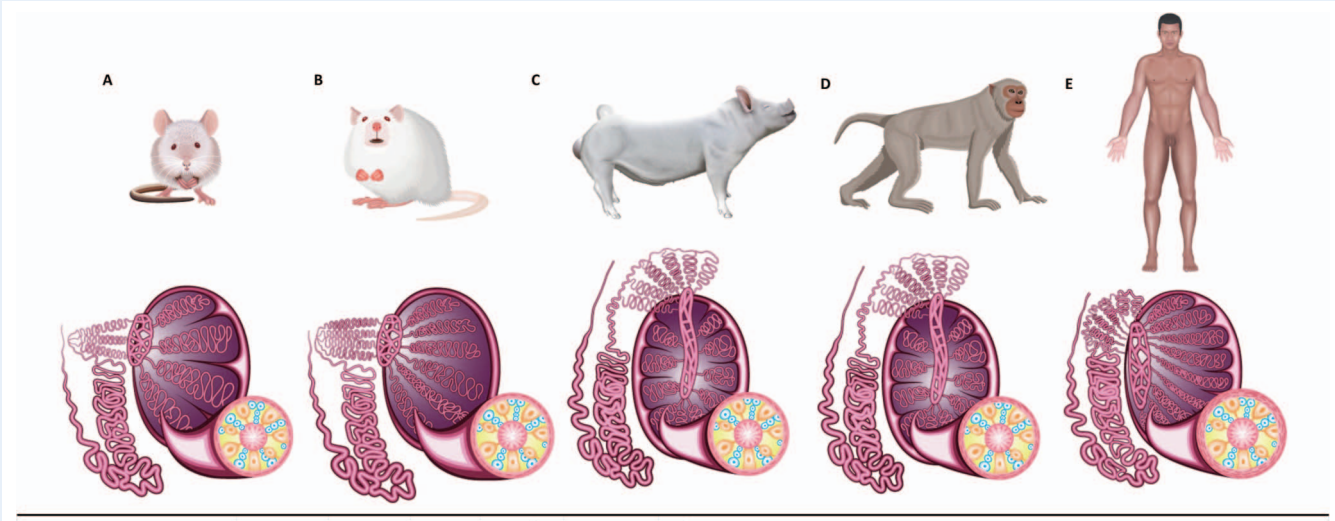
The wall thickness of the seminiferous tubule may be a highly relevant parameter to a transplantation procedure in regard to the potential increment of the intratubular pressure induced by injection, which could result in rupture of the membrane. Flattened myoid cells in the lamina propria, which encircles the seminiferous tubule together with the basement membrane, are thought to promote the movements of the spermatozoa into the lumen of the testis via contractile activity. The connective tissue lamellae is thicker and has more layers of flattened myoid cells in humans than in other species (Wing and Christensen 1982; Wing and Christensen 1982; Pinart *et al*., 2001; Wistuba *et al*., 2007; Bonet *et al*., 2013; O'Shaughnessy 2014; Treuting *et al*., 2017) (Fig. 2). This suggests that the thicker human lamina propria may be more resistant to intratubular microinjections, which may hamper the success of the transplantation procedure (Schlatt *et al*., 1999). In line with this, Johnson *et al*. (1980) demonstrated that although human seminiferous tubules are smaller than the tubules of rats, the tubular boundary tissue (basement membrane and lamina propria) of the human tubules was much thicker compared to the rats (Johnson *et al*., 1980). In the interspace between the myoid cells, layers of collagen fibres can be observed (Hermo *et al*., 1977; Pinart *et al*., 2001; Bonet *et al*., 2013; Creasy and Chapin 2018). The increased thickness of the layer of collagen fibres, peritubular fibrosis and hyaline development can be observed upon seminiferous tubule damage and aging, which may alter the success rates of SSC transplantation (de Kretser *et al*., 1982; Pop *et al*., 2011; Creasy and Chapin 2018).

Rete testis

The terminal channels of the seminiferous tubules dilate and form straight tubules. These straight tubules comprise the transitional component of the seminiferous tubules, as they connect with a network of channels gathering into a structure termed the rete testis. This is where the spermatozoa are mixed with luminal contents and then accumulate (Roosen-Runge and Holstein 1978). Some studies have described a sudden narrowing of the tubular diameter in the connection of the convoluted seminiferous tubules and straight tubules, suggesting that the narrowing may be a sphincter for avoiding the backflow of the spermatozoa (Dym 1976; Roosen-Runge and Holstein 1978; Silván and Aréchaga 2012). This narrowing may require some transplantation modifications, such as modifying the pressure, and may alter the success rates of the SSC transplantation procedure. The rete testis' ducts occupy a rather extensive region of the testis, normally described as a flattened cavernous space that is more dilatated than the seminiferous tubules due to their role in the mixture and transportation of the seminiferous tubule fluid accompanied by the spermatozoa outside the testis (Dym 1976; Roosen-Runge and Holstein 1978; Saitoh *et al*., 1990; Silván and Aréchaga 2012; Bonet *et al*., 2013; Pereira Bacares *et al*., 2017; Creasy and Chapin 2018).

The human rete testis is connected to the margin within the testicular hilum adjacent to thickened connective tissue. In contrast, the rodent rete testis (mice, rats, hamsters) is positioned in a more subcapsular (superficial) position compared to humans, boar and bull, which makes it an ideal and accessible site for the SSC transplantation of rodents (Treuting *et al*., 2017) (Fig. 2). In other species, including rabbit, guinea pig, cat, dog, ram, bull, boar and monkey, the rete testis is positioned longitudinally in the centre of the testis (Dym 1976; Creasy and Chapin 2018) (Fig. 2). The difference in the location of the rete testis is striking in regard to injection techniques, because the rete testis in the bull, boar, monkey and human (more proximal to the epididymis) is located more centrally than in rodents (Sinowatz *et al*., 1979; Ilio and Hess 1994; Creasy and Chapin 2018) (Fig. 2). Since the rete testis can be more difficult to visualize and access in these species than in rodents, the aid of imaging techniques such as ultrasonography is required (Saitoh *et al*., 1990; Lambot *et al*., 2009; Silván and Aréchaga 2012; Nakata *et al*., 2015).

According to Ogawa *et al*. (1997), injection of SSCs into the rete testis could be beneficial, as the rete testis already is responsible for the collection and mixture of the seminiferous fluid and mature spermatozoa, prior to its passage through the extratesticular ducts (efferent ducts, epididymis and ductus deferens) (Ogawa *et al*., 1997). However, the intratesticular rete has very thin and fragile walls, which



	Mouse	Rat	Boar	C. Monkey	Human	References
Testis weight *(g)*	0.03-0.08	1-2.4	64-360	16-25	15-22	Treuting et al. (2017); Breed (1998); Bonet et al. (2013); Almeida et al. (2006); Haruyama et al. (2012), Mirsky et al. (2016); Dixson (2013); Zhengwei et al.(1998); Johnson et al. (1984)
Daily sperm output *(x 10^6/g)*	54	21	29	23	3.1-4.25	Creasy and Chaplin (2018); Almeida et al. (2006)
No. of seminiferous tubules	7-11	30	ND	200	400-800	Breed (1998); Nakata et al. (2015); Wing and Christensen (1982); Wistuba et al. (2003)
Luminal diameter of the seminiferous tubule *(μm)*	175	292	240	210	220	Mehraein and Negahdar (2011); Johnson et al. (1980); Wing and Christensen (1982); Ma et al. (2016); Bonet et al.(2003); Almeida et al.(2006); Zhengwei et al. (1997); Zhengwei et al. (1998); Haruyama et al. (2012); Johnson et al. (1980); Holstein et al. (2003)
Volume density of seminiferous tubules (%)	90	58-90	87	31	41-51	Breed (1998); Johnson et al. (1980); Almeida et al.(2006); Dixson (2013); Johnson et al. (1980)
Thickness of lamina propria *(μm)*	ND	ND	4-5	ND	4-8	Bonet et al.(2013); Pop et al.(2011)
Width of the lumen rete *(mm)*	0.5	1.3	ND	ND	3	Silván and Aréchaga (2012)
No. of efferent ducts	2-5	5-9	12-14	8-16	5-15	Silván and Aréchaga (2012); Nakata et al. (2015); Treuting et al. (2017); Jones and Jurd (1987); Stoffel and Friess (1994); Ramos and Dym (1977); Roosen-Runge and Holstein (1978); Saitoh and Hatekeyama (1990); Wistuba et al. (2003)
Luminal diameter of the efferent duct *(μm)*	50	94	240	ND	100-150	Silván and Aréchaga (2012); Oliveira et al. (2002); Stoffel and Friess (1994); Saitoh and Hatekeyama (1990)
Lobule number	7-11	No lobule	200	200	200-300	Johnson et al. (1980); Wing and Christensen (1982); Holstein et al. (2003); Bonet et al.(2013); Creasy and Chapin (2018); Maynard and Downes (2019)
Layers of flattened myoid cells	Single	Single	Single	1-3	2-6	Hermo et al. (1977); Wing and Christensen (1982); Pinart et al. (2001); Wistuba et al. (2007); Bonet et al. (2013); O'-Shaughnessy (2014); Treuting et al. (2017)

Figure 2 Morphological and stereological parameters of the testis architecture among four selected relevant mammalian species. The illustration shows five different species the (**A**) mouse (*Mus musculus*), (**B**) rat (*Rattus rattus*), (**C**) boar (*Sus scrofa*), (**D**) cynomolgus monkey (*Macaca fascicularis*) and (**E**) human (*Homo sapiens*), representing the diversity of testis architecture and interspecies comparison of the morphological and stereological parameters of the testis. ND: not determined; C. monkey: cynomolgus monkey. All data presented in this table are mean values.

are easy to penetrate and therefore may lead to stromal contamination (Silván and Aréchaga 2012). In this regard, Silván *et al*. (2012) suggested that the micropipette must be inserted parallel to the surface of the testis for SSC transplantation (Silván and Aréchaga 2012), a particularly challenging feat due to the central position of the rete testis.

Although injection of SSCs into the human rete testis will be challenging, the rete testis consists of three components: septal rete, mediastinal rete and extratesticular rete (Roosen-Runge and Holstein 1978). The last component, the extratesticular rete, comprises a dilatation of the channels, representing the vestibules for the efferent ducts (see below). This portion of the human rete testis might be an optimal site for transplantation given its greater diameter when open surgery is considered.

Efferent ducts

From the rete testis, the spermatozoa are transported outside of the testis via the extratesticular rete and the efferent ducts. Efferent ducts arise from the dilated extratesticular rete testis to connect to a single tubular structure, the ductus epididymidis. A number of ducts (between 2 and 16 depending on the species) penetrate the tunica albuginea, coil and approach to form the head of the epididymis (Dym 1976; Ramos and Dym 1977; Jonte and Holstein 1987; Saitoh *et al*., 1990; Stoffel and Friess 1994; Treuting *et al*., 2017) (Fig. 2).

Morphological analyses of the branching patterns of mammalian efferent ducts show that there is considerable variation between the species (Ilio and Hess 1994). The two basic designs have been described in terms of the mammalian efferent ducts themselves as well as their connection to the rete testis and epididymis. The first design is typical in rodents, in which the efferent ducts anastomose to form a funnel-like structure that ends up in a single tubule, called the common efferent duct. The common efferent duct connects with the epididymal head (Dym 1976; Lambot *et al*., 2009) (Fig. 2). In larger mammals, the second design involves parallel efferent ducts with multiple entries into the head of the epididymis (Dym 1976) (Fig. 2). In this case, some of the efferent ducts anastomose before connecting with the epididymal ducts (Saitoh *et al*., 1990; Ilio and Hess 1994) (Fig. 2); however, the importance of these designs in regard to SSC transplantation is unknown.

Although the importance of efferent duct design for SSC transplantation is currently unclear, there are implications for surgical strategies. Rodents have more tortuous and long efferent ducts, all located within

the epididymal fat pad (Silván and Aréchaga 2012; Treuting et al., 2017; Creasy and Chapin 2018). Therefore, fat tissue dissection should be the first step in SSC transplantation in rodents. In contrast, efferent ducts are much shorter and located proximally close to the epididymal head in humans suggesting that fat tissue dissection would be minimal (Creasy and Chapin 2018). Moreover, the wall of the efferent ducts, the epididymal duct and deferent duct are considerably thicker than the rete testis, due to the presence of additional layers of myoid cells, which are principally arranged in a spherical manner (Roosen-Runge and Holstein 1978; Creasy and Chapin 2018). These features may make efferent ducts a possible candidate for SSC transplantation in human; however, this would require an open surgical procedure.

SSCs: from rodent to human

As our knowledge of SSCs grows, one of the biggest obstacles to translation into human and clinical treatment is the selection of the appropriate SSC. After puberty, highly active spermatogenesis is maintained by SSC self-renewal and continuous differentiation into spermatozoa (Kubota and Brinster 2018). The identification of the proper SSC from rodents, domestic animals or primates has involved several different experimental approaches in the last half century; however, markers specific to SSC or specified for the SSC population within the subset of spermatogonia remain elusive (Sharma et al., 2019). In rodent animals, such as mouse and rat, spermatogenic differentiation is followed by mitotic divisions from A_{single} to A_{pair}, $A_{aligned}$ and A1–A4 spermatogonia. Type B and intermediate spermatogonia are formed by continuous mitotic expansion (Ehmcke et al., 2006). The number of SSCs is estimated to be only 0.01% in adult mouse testis, as recorded by a transplantation assay (Nagano 2003). A_{single} spermatogonia are generally considered to be SSCs; however, the employment of a green fluorescent protein (GFP)-labelled, transgenic mouse model combined with live imaging studies identified that $A_{aligned}$ spermatogonia may contain a small fraction of SSCs, as A_{pair} and A_{single} can be generated from the fragmentation of $A_{aligned}$ spermatogonia (Hara et al., 2014). Several approaches to enrich SSCs obtained from postnatal mice have been developed, such as density-gradient centrifugation, differential plating, created cryptorchidism and cell surface marker-based selections (Shinohara et al., 1999; Shinohara et al., 2000a; Kubota et al., 2004). Currently, the most precise method for isolating SSCs is fluorescence-activated cell sorting (FACS). For example, the cluster of differentiation 90 positive ($THY1^+$), integrin subunit alpha 6 positive, cluster of differentiation 117 negative, MHC class I negative cell population has a 300-fold higher SSC concentration than unfractionated adult testis cells (Kubota et al., 2003). In addition, integrin beta-1 (ITGB1), CD9, glial cell line-derived neurotrophic factor (GDNF) family receptor alpha-1 (GFRA1), epithelial cell adhesion molecule, cluster of differentiation 24, cadherin-1 and melanoma cell adhesion molecule are also enriched in undifferentiated spermatogonia (Shinohara et al., 1999; Shinohara et al., 2000b; Kubota et al., 2003; Kanatsu-Shinohara et al., 2004; Kanatsu-Shinohara et al., 2012b). Using GFP reporter mice, octamer-binding transcription factor 3/4, neurogenin-3, putative homeobox protein NANOG2 (Nanog2), inhibitor of DNA binding 4 (Id4), polycomb group RING finger protein 4, paired box protein 7 and telomerase reverse transcriptase are also expressed in undifferentiated spermatogonia (Ohbo et al., 2003; Ohmura et al., 2004; Yoshida et al., 2004; Sada et al., 2009; Aloisio et al., 2014; Chan et al., 2014; Komai et al., 2014; Pech et al., 2015); however, a SSC-specific marker remains to be identified via functional assays, flow cytometry and live imaging. In domestic animals, the phenotypic markers of spermatogonia and the characterization of SSC are both relatively rare. Ubiquitin carboxy-terminal hydrolase L1 (UCHL1) was seen to be expressed in mouse spermatogonia (Kon et al., 1999), and UCHL1 is also found in premeiotic male germ cells in pigs, cattle, buffalo and goats. UCHL1, zinc finger and BTB domain-containing protein 16, THY1, GFRA1 and Nanog2 are all suggested to be expressed in spermatogonia (Zheng et al., 2014); however, the molecular SSC-specific marker in domestic animals is still elusive. In primates, two types of Type A spermatogonia, namely A_d spermatogonia and A_p spermatogonia, were described by Clermort (1970). A transiting spermatogonial population called $A_{transition}$, which is morphologically intermediate and distinct from A_d and A_p spermatogonia, constitutes 25 to 50% of type A spermatogonia (Ehmcke et al., 2006). SSCs are believed to be a subpopulation of type A spermatogonia in primates; however, the evaluation of enriched SSCs is still unclear (Di Persio et al., 2017). Recently, the heterogeneity of undifferentiated human spermatogonia was investigated by four independent groups using a single-cell RNA sequencing approach (Guo et al., 2018; Hermann et al., 2018; Wang et al., 2018; Sohni et al., 2019). Cluster analysis revealed 3, 5, 7 or 10 sub-clusters (potentially different types) of adult spermatogonia. Pseudotime trajectory analysis identified the most primitive spermatogonia, which were assumed to be SSC in adult testis. This included the gene expression of ID4, ZBTB16, GFRA1, FGFR3 and UTF1, which have been proposed to be SSC markers in both mouse and human SSC (von Kopylow and Spiess 2017). One major complication, however, is that all those markers are not only found in the primitive spermatogonia cell clusters but also in advanced and even differentiated spermatogonia by single-cell RNA sequencing (Tan and Wilkinson 2019). Therefore, the definition of any SSC-specific markers in human remains obscure. Identification of SSC-specific transcripts is not the only issue facing the SSC field. Sohni et al. identified LPPR3 and TSPAN33 as being highly enriched in primitive, undifferentiated spermatogonia (Sohni et al., 2019); however, functional assessments of SSC activity, such as xenotransplantation, to provide definitive evidence of SSCs are still lacking (Fayomi and Orwig 2018).

SSC transplantation in animals

The promise of SSC transplantation to restore spermatogenesis and fertility is based on several lines of evidence. SSC transplantation restoring spermatogenesis in infertile individuals was initially demonstrated in mouse. Later, this concept was applied to many other mammals, including dog, pig and cattle (Table I). Currently, offspring have been born from donor-derived sperm from mice, rat, sheep, goat and treeshrew after SSC transplantation. The SSC transplantation techniques were developed for different species according to their corresponding testicular architectures.

Rodents

Fertility restoration via SSC transplantation was first established in mice (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). The seminiferous tubule, efferent duct and rete testis were suggested

Table I Characteristics of studies based on *in vivo* spermatogonial stem cell transplantation in animal testes.

Animals	Injection sites	Injection volume per testis	Injection cell concentration per testis	Current state of success	References
Mouse	Efferent duct Seminiferous tubules. Rete testis	Around 10 µl with (10–30 µl)	5–300 × 10^6 cells/ml	Colonization, spermatogenesis and offspring generation	(Brinster and Avarbock 1994; Ogawa *et al*., 2000; Shinohara *et al*., 2001; Kanatsu-Shinohara *et al*., 2003a; Shinohara *et al*., 2006; Kanatsu-Shinohara *et al*., 2016)
Rat	Rete testis	Around 65 µl (10–100 µl)	Around 1 × 10^6 cells/ml	Colonization, spermatogenesis and offspring generation	(Ogawa *et al*., 1999; Ryu *et al*., 2003; Zhang *et al*., 2014; Chapman *et al*., 2015)
Dog	Rete testis	1 ml	1.2 × 10^6 cells/ml	Colonization, spermatogenesis	(Kim *et al*., 2008, Harkey *et al*., 2013)
Ram (goat)	Rete testis	5 ml	6–135 × 10^6 cells/ml	Colonization, spermatogenesis and offspring generation	(Honaramooz 2003a, Honaramooz *et al*., 2003b, Kaul *et al*., 2010)
Ram (Sheep)	Rete testis	5 ml	30–40 × 10^6 cells/ml	Colonization, spermatogenesis and offspring generation	(Rodriguez-Sosa *et al*., 2006; Herrid *et al*., 2009; Herrid *et al*., 2009; Herrid *et al*., 2011; Stockwell *et al*., 2013; Kojima *et al*., 2017)
Boar	Rete testis	3–15 ml	2.3–75 × 10^6 cells/ml	Colonization, spermatogenesis and *in vitro* fertilization of oocytes	(Honaramooz *et al*., 2002; Mikkola *et al*., 2006; Zeng *et al*., 2013; Lin *et al*., 2017)
Bull	Rete testis	1–5 ml	5–25 × 10^6 cells/ml	Colonization, spermatogenesis	(Izadyar *et al*., 2003; Herrid *et al*., 2006; Stockwell *et al*., 2009)
Treeshrew	Efferent ducts	Around 15 µl	3–5 × 10^6 cells/ml	Colonization, spermatogenesis and offspring generation	(Li *et al*., 2017)
Monkey (cynomolgus monkey)	Rete testis	100–200 µl; 2000–3000 µl	NR	4 of 5 monkeys testis regrowth	(Schlatt *et al*., 1999; Schlatt 2002)
Monkey (Rhesus Macaque)	Rete testis	150–400 µl for prepuberty and puberty Around 1 ml for adult	58–232 × 10^6 cells/ml	Colonization, spermatogenesis and IVF of oocytes	(Jahnukainen *et al*., 2011a; Hermann *et al*., 2012)

NR: not reported

injection sites for SSC transplantation (Ogawa *et al*., 1997; Goodyear and Brinster 2017). Injection into a single seminiferous tubule limited the delivery of SSCs into the entire tubular network since there are many individual tubules with both ends connected to the rete testis. On the other hand, injection into rete testis caused a leakage of the transplanted suspension into the interstitium due to the difficulty in controlling the injection angle and depth into the rete testis. Injection through an efferent duct seems to be the most efficient technique in mice with a filling rate of 70 to 100% of surface tubules (Medrano *et al*., 2014); however, all described transplantation methods in mouse supported the regeneration of spermatogenesis (Ogawa *et al*., 1997).

SSC transplantation in mice has become the gold standard for investigating the functionality of putative SSCs (Goodyear and Brinster 2017; Takashima and Shinohara 2018). Intratesticular application of busulfan is a safe and effective way to deplete endogenous spermatogenesis prior to transplantation in mice (Qin *et al*., 2016a; Qin *et al*., 2016b). SSC transplantation in combination with virus infection (Nagano *et al*., 2001), plasmid transfection (Kanatsu-Shinohara *et al*., 2005), transcription activator-like effector nucleases technology (Sato *et al*., 2015) and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 technology (Chapman *et al*., 2015) (these technologies allow genome editing in DNA sequence) also generated transgenic offspring in mice (Table I).

However, since rats more clearly reflect human physiology and mimic human diseases, such as male reproductive system aging and immune response, than mice (Wang *et al*., 2002; Wildner 2019), SSC transplantation into the rat testis has also been intensively investigated (Ogawa *et al*., 1999). Most researchers have transplanted stem cells into rat seminiferous tubules via injection through the rete testis (Ryu *et al*., 2003; Chapman *et al*., 2015) (Table I).

Domestic animals

SSC transplantation has also successfully restored spermatogenesis in several domestic animals, such as dog, boar, goat and cattle (Kubota and Brinster 2018). The dog is a desirable model for preclinical

translational research due to its physiology, lifespan and genetics that match the human parameters more closely than those of mice and rats. Moreover, experiments using dogs are more cost-effective than those with primates (Tsai et al., 2007). SSC transplantation after irradiation of the testes has successfully generated haploid gametes in dogs (Kim et al., 2008; Harkey et al., 2013). The SSCs were transplanted via injection of rete testis using a 22-G i.v. catheter inserted through the caudal pole of the testis, under the guidance of ultrasonography. Injections led to spermatogenesis in 5% of the seminiferous tubules, whereas the injection of fresh cells resulted in spermatogenesis in 15% of the seminiferous tubules (Kim et al., 2008) (Table I).

Honaramooz and colleagues (Honaramooz 2003a; Honaramooz et al., 2003b) were the first to perform SSC transplantation in prepubertal goats. They identified the optimum transplantation site for SSCs and revealed that efferent duct transplantation is far less practical than rete testis injections, since the dissection of epididymis to visualise the efferent ducts is complicated and time-consuming. Therefore, for transplantation, the injection needle was passed through the ventral scrotal skin to enter the epididymis and then into the rete testis of untreated goat testis under the guidance of ultrasonography. The injection was conducted with a 20-G i.v. catheter with an infusion rate of 0.5–1 mL/min. This resulted in the filling up of 20% of the seminiferous tubules with dyed donor cells. Using this technique, the injection of germ cells successfully transmitted the donor haplotype to the next generation (Honaramooz et al., 2003b). Using the same transplantation method and site described by Honaramooz et al., (2003b), Kaul and co-workers observed a similar filling efficiency (20 to 30% of the seminiferous tubules) in goat, with higher concentrations of injected cells (Kaul et al., 2010) (Table I).

The efficiency of SSC transplantation through the rete testis was also demonstrated in ram *ex vivo* and *in vivo* (Rodriguez-Sosa et al., 2006). In this study, four different transplantation methods were tested *ex vivo*: efferent duct with an extratesticular rete connection (20 G); efferent duct with an extratesticular rete connection with a larger needle; proximal end of the mediastinum testis (intratesticular rete); and distal end of the mediastinum testis (intratesticular rete). The second, third and fourth approaches were performed under the guidance of ultrasonography with a 26-G needle. In the *in vivo* transplantation, a two-needle system was used, as a 20-G needle was initially injected through the scrotum and into the extratesticular rete testis under the guidance of ultrasonography. Then, a second 26-G needle was inserted through the 20-G needle. Injections were performed via gentle pressure on the syringe until resistance was observed. In other studies, authors used higher concentrations with a 20-G catheter into the rete testis of irradiated testes of ram for SSC transplantation (Herrid et al., 2009; Herrid et al., 2011; Stockwell et al., 2013). Successful injection was confirmed by the observation of a small quantity of injected air using ultrasonography.

Boar, another domestic animal, was also investigated for SSC transplantation. Rete testis injections with the aid of ultrasonography led to a filling efficiency of up to 50% of the seminiferous tubules (Honaramooz et al., 2002). Mikkola et al. (2006) used an 18-G needle to transfer a cell suspension via injection through the scrotal skin into the rete testis of boars diagnosed with hereditary immotile short-tail sperm defects. They used ultrasonography guidance with a flow rate of 0.5–1 mL/min. Three months after the SSC transplantation, the first motile spermatozoa were observed in the ejaculates of the boar. Similarly, Zeng et al. successfully performed SSC transplantation in pigs exposed to busulfan during foetal development (Zeng et al., 2013). They used a 20-G needle inserted through the cauda epididymis and testis into the rete testis under ultrasonography guidance to transplant the SSCs in prepubertal boars. When pigs became sexually mature, the mature spermatozoa were collected from semen and used for IVF to produce embryos.

Currently, the concept of SSC transplantation has been implemented in cattle production systems (Oatley 2018) to provide a breeding tool for the introgression of genetic traits (Oatley 2010). Izadyar et al. (2003) performed SSC transplantation into the rete testis of irradiated bovine testis using an 18-G needle under the guidance of ultrasonography and observed a complete regeneration of spermatogenesis 2.5 months after transplantation. In another study, bull spermatogonia were transplanted into the rete testis by injecting a cell suspension at a rate of 5 mL/1–2 min (Herrid et al., 2006). Donor cells were found in 50% of the recipient testes for up to 6 months. A similar injection was performed by successfully placing a catheter in the rete testis of untreated recipients (Stockwell et al., 2009). After 1 year of transplantation, donor DNA was observed in both single-cell suspensions and testis tissue from this recipient. In summary, SSC transplantation into the rete testes of several domestic animals has successfully restored spermatogenesis.

Non-human primates

Non-human primate studies provide invaluable insight into clinical transplantation (Phillips et al., 2014). Currently, there are four studies on SSC transplantation in non-human primates (Schlatt et al., 1999; Schlatt 2002; Jahnukainen et al., 2011a; Hermann et al., 2012) (Table I). The first report on germ cell transplantation in monkeys was presented in 1999 (Schlatt et al., 1999). The testes of two cynomolgus monkeys treated with a GnRH antagonist before transplantation were injected with a 2- to 3-mL cell suspension through the rete testis. Four weeks following transplantation, histological analysis revealed the presence of labelled cells in some seminiferous tubules at the base of the epithelium and in the interstitium. Subsequently, SSC transplantation was performed on five adult male cynomolgus monkeys whose testes were treated with irradiation before transplantation (Schlatt 2002). A small volume of cell suspension (100–200 µl) was injected through the rete testis from the caudal pole under ultrasonography guidance. The total procedure time was 20–30 min for both testes under general anaesthesia. Six weeks after transplantation, the volume of the injected testes was increased in 60% of the recipients. A decade later, two pubertal and four prepubertal macaque monkeys were exposed to testicular irradiation prior to germ cell transplantation (Jahnukainen et al., 2011a). Two months after irradiation, cryopreserved germ cells were transplanted autologously through the scrotum into the rete testis, without ultrasonography guidance. Injections into the rete testis of immature monkeys revealed lower back pressure and a higher resistance of rete testis tissue compared with the surrounding tubular tissue. Only one of the four prepubertal monkeys showed enhanced spermatogenesis (Jahnukainen et al., 2011a). These two studies indicated that optimization of the transplantation techniques is a critical step in achieving successful outcomes (Schlatt et al., 2002; Jahnukainen et al., 2011a). Hermann et al. (2012) were first to show functionality after SSC transplantation in the monkey. In these experiments, busulfan

treatment was implemented to deplete the endogenous spermatogenesis of 12 adult and five prepubertal rhesus macaques. The SSC transplantation procedure was performed via ultrasound-guided rete testis injections using a 25-G spinal needle under slow, constant pressure. An average of 1041 ± 82 and 222 ± 26 µl of cell suspension was introduced to the rete testis of adult and juvenile recipients, respectively. An average of 88 million viable cells were injected into each adult testis, while the average cell concentration injected into the juvenile testis was 45.8 million viable cells. Spermatogenesis was observed in 11 out of 12 adults and all prepubertal recipients after transplantation. Ejaculated donor-derived sperm were observed in 9 out of 12 adults and three out of five prepubertal recipients after reaching maturity. Donor-derived sperm cells were capable of fertilizing 81 out of 85 oocytes and 7 out of 81 embryos were further confirmed to have donor paternal origins (Hermann *et al*., 2012). The results encourage clinicians and scientists to develop a SSC transplantation-based therapy to restore fertility in humans.

SSC transplantation in humans

SSC transplantation is a potential method for fertility restoration in patients undergoing gonadotoxic treatments for their condition or who have benign diseases that may cause infertility. To translate this approach to the clinic, the optimization of SSC transplantation is essential. Radford *et al*. (1999) were the first to inject SSCs *in vivo* through the testes of five adult male cancer survivors whose testicular tissues had been collected and cryopreserved as single-cell suspensions prior to gonadotoxic treatment (Radford *et al*., 1999; Radford 2003). However, because the researchers did not publish the outcomes of their study, detailed information on their transplantation procedure is unavailable. Nevertheless, in that study, patients were willing to participate in experimental options of fertility management in order to have their own genetic children even though fertility was not guaranteed. Considerations involved in the clinical application of SSC transplantation in human testes, such as the best injection site for SSC transplantation, the sufficiency of a single infusion, most efficient injection volume and cell concentration and infusion depth, are still unanswered.

The best injection site for SSC introduction: the rete testis

As with all other interventional procedures, SSC transplantation should be safe and kept as simple, effective, rapid and inexpensive as possible. After the proof-of-concept study by Brinster and Zimmerman (1994), three sites were described to introduce germ cells into mouse testis: efferent ducts, rete testis and seminiferous tubules (Ogawa *et al*., 1997). Regardless of the species, efferent duct injection has been seen as the most compelling technique in terms of preparation, localization and cannulation (Schlatt *et al*., 1999; Ma *et al*., 2013). Injections through the efferent duct have resulted in promising outcomes in animals with small testes; however, this approach was reported as impractical in species with larger testes, such as monkeys and bulls (Hermann *et al*., 2012; Giassetti *et al*., 2019). It has also been reported that it is too difficult to dissect the epididymis from the apical pole of the testis due to the interwoven structures of fatty and connective tissues (Hess 2015). A morphological study investigating the human testis found that efferent ducts are highly coiled and tightly packed within in a small space (Saitoh *et al*., 1990), making it an unlikely site for SSC transplantation.

The first *ex vivo* effort to identify the best injection route for human testes was conducted in 1999 (Schlatt *et al*., 1999). Isolated germ cells from rats were injected with trypan blue dye into human cadaver testes through three different areas: the seminiferous tubules, the efferent ducts and the rete testis. Following the efferent duct injections, a macroscopic evaluation of the human testes revealed no trypan blue dye or germ cells in the seminiferous tubules. However, the full length of the rete testis and nearby seminiferous tubules could be filled with dye via rete testis injection once the needle was placed into the correct injection site. Taken together, microinjection into both the efferent ducts and seminiferous tubules was impractical due to resistant lamina propria and the convoluted structure of the seminiferous tubules (Schlatt *et al*., 1999).

The most challenging component of rete testis injection has been the placement of the needle. To overcome this issue, Schlatt and co-workers performed injections through the rete testis using one cynomolgus monkey (*in vivo*) and four human testes (*ex vivo*) under ultrasonography guidance (Schlatt *et al*., 1999). In the monkey, complete filling of the rete testis and a few seminiferous tubules was observed, while similar findings in the rete testis, with a variable extent of tubular infusion, were observed in the human testes. The authors suggested that ultrasound-guided rete testis injection was the most favourable approach to SSC transplantation, as it provided a more intensive infiltration of the seminiferous tubules, and larger volumes could be delivered into the testes (Schlatt *et al*., 1999). However, in this study, the rate of successful attempts at rete testis injections was only 33%, and only a limited number of seminiferous tubules around the rete testis contained injected cells.

Similarly, Ning *et al*. (2012) attempted to identify a more efficient site for human SSC transplantation *ex vivo* (Ning *et al*., 2012). They infused a single dose of a solution (800 µl) at a constant hydrostatic pressure into four different sites (deferent duct, head of the epididymis, rete testis and blind infusion) of 12 human cadaver testes. The rete testis injections were performed using ultrasonography guidance. The ultrasonography showed lucid fluid streams during injection through the rete testis into the testis parenchyma. Thirty minutes after injection, they performed computerized tomography (CT) scans to confirm the spreading of the infused solution. During histological analysis, ink particles were observed in the rete testis and seminiferous tubules; however, they were not observed in the seminiferous tubules of testes injected via the epididymis and deferent duct (Ning *et al*., 2012). Ink particles were also not observed in the seminiferous tubules following the blind infusions.

Although there are no other human studies comparing the efficiencies of different puncture sites, the efficacy and feasibility of rete testis injections on human cadaver testes were confirmed by several *ex vivo* studies. For example, Brook *et al*. (2001) endeavoured to infuse eight human cadaver testes (Brook *et al*., 2001). Although they did not use ultrasonography guidance for the rete testis infusions and did not compare the different puncture sites, macroscopic evaluation of the seminiferous tubules revealed that they were filled with an average efficiency of 55% by rete testis infusion (Brook *et al*., 2001). Faes and co-workers (2013) injected 800 µl of radioactive cell suspension into six human cadaver testes to test the feasibility of the ultrasonography-guided rete testis injection method under constant hydrostatic pressure

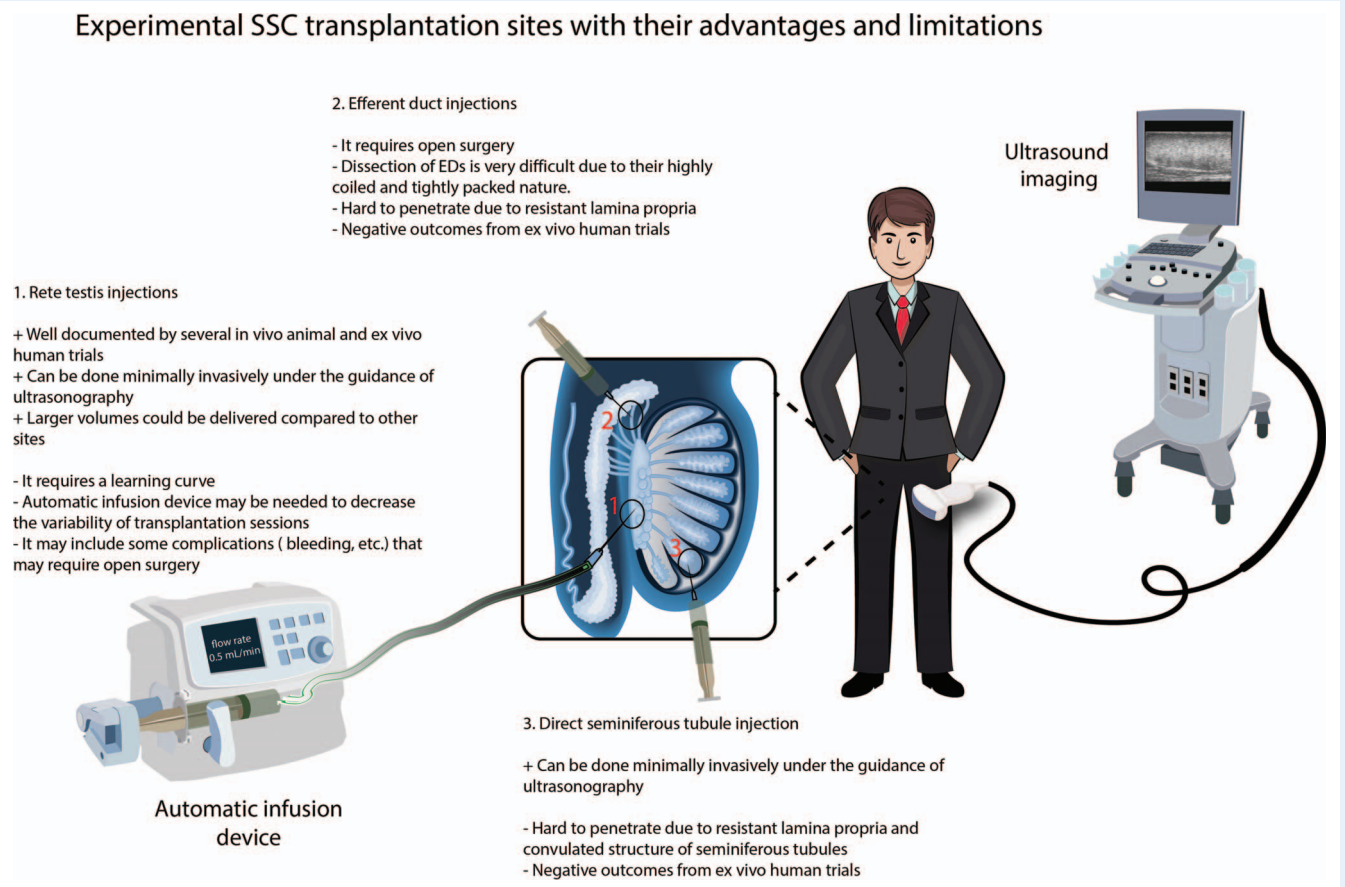


Figure 3 Experimental SSC transplantation sites with their advantages and limitations. ED: efferent duct; RT: rete testis; ST: seminiferous tubule. Using ultrasonography and an automatic infusion pump during rete testis injections showed a better success rate in animal and human studies.

(Faes *et al*., 2013). Single-photon-emission-computerized tomography (SPECT) confirmed the efficiency of this method. Histological analysis also confirmed that 20% of the seminiferous tubules were filled with GFP-positive (GFP^+) donor cells (Faes *et al*., 2013). Similarly, in another *ex vivo* study, SSC transplantation through the rete testis was proven to be efficient, even at different levels of pressure (Faes *et al*., 2017). Combined with the other animal studies on larger testes, current evidence from human studies demonstrates that ultrasonography-guided rete testis injection seems the most plausible, feasible, effective and minimally invasive method of any other methods for SSC transplantation. Critically, injection accuracy was directly associated with the success of SSC transplantation, suggesting that this procedure will have a learning curve. However, Doppler ultrasonography has to be used to increase the accuracy of the injection procedure and the use of an automatic infusion pump has also been proven to lessen the variability in transplantation sessions. Hence, the development of automatic infusion devices to eliminate the learning curve and increase SSC transplantation efficiency is essential (Fig. 3).

Single or multiple infusions?

Although the relevant studies to date have shown that rete testis injection is the best way to introduce SSCs into the human testis, Brook *et al*. (2001) were the first to associate the increased efficacy of rete testis injection with multiple infusions (Brook *et al*., 2001). To achieve this, 200 µl of trypan blue dye or red blood cells in heparinized phosphate-buffered saline (PBS) was injected through the rete testis either at a single site or at four sites along the length of the rete testis as multiple injections at 75 cm of hydrostatic pressure (Table II). After infusion, the testes were immediately incised and examined for trypan blue dye/erythrocyte distribution and affected seminiferous tubules. Multiple infusions were found to be more efficient than a single-site injection (Brook *et al*., 2001); however, these results were based only on macroscopic observations and not histological evaluations. Ning *et al*. were the first to evaluate the effect of multiple-site infusions via histological and imaging methods (Ning *et al*., 2012). In their study, a multiple-site infusion technique was implemented through the rete testis of eight human testes, and a single 800-µl infusion method was compared with two 400- and four 200-µl infusions (Ning *et al*., 2012). They were unable to demonstrate the advantage of multiple- over single-site injections via the CT results. The histopathologic examination also revealed that multiple injections resulted in more leakage to the interstitial compartment than filling the tubular compartment (Ning *et al*., 2012). Although it is very difficult to draw a conclusion due to the lack of multiple infusion studies, current evidence does not support improved outcomes from multiple infusions through the rete testes.

Table II Characteristics of studies based on spermatogonial stem cell transplantation in human testes.

Study	Year	Subjects	Time elapsed tissue resection to transplantation	Injection area and used the needle	Injection pressure	Injected material	Injection time	Injected volume/cell concentration
Radford *et al.*	1999	5 men with various cancer (*in vivo*)	NR	NR	NR	Cryopreserved single cell suspension (isolated from testicular tissue biopsy)	NR	NR
Schlatt *et al.*	1999	6 testes from men with prostate cancer undergoing orchidectomy (*ex vivo*)	NR (all samples were kept on ice before the injection)	ST—30-40 µm ED—30-40 µm RT with US—23 gauge	low HP *	Trypan blue dye + testicular cell suspension isolated from rats	30 min	<50 µl for ST, <100 µl for ED *and* 2–5 ml for RT with US
Brook *et al.*	2001	8 testes from men with prostate cancer undergoing orchidectomy (*ex vivo*)	NR (refrigerated for up to 24 h before transportation)	RT without US—single injection or multiple injections	75 cm HP	Trypan blue dye solution *or* Red blood cells(erythrocytes)	NR	200 µl at either a single dose *or* at four points through RT
Ning *et al.*	2012	20 cadaver testes from men autopsied at the department of pathology (*ex vivo*)	Testes were isolated 3 h after death and injected and fixed within 2 h after isolation	RT with US—23 gauge Single or multiple infusions	75 cm HP	Chinese ink + US contrast + CT contrast (no cells)	2.5–4.5 min	800 µl (single) *or* 2 × 400 µl *or* 4 × 200 µl
Faes *et al.*	2013	18 cadaver testes from men autopsied at the department of pathology (*ex vivo*)	The average ischemia time from cardiac arrest to the time of infusion was 27 ± 13 h. (13–55 h)	RT with US—23 gauge	75 cm HP	^{99m}Tc-labelled GFP^{+} testicular cells isolated from mice + Microbubbles	1–2 min	800 µl with 20 million cells/ml ($n = 6$) *or* 800 µl with 10 million cells/ml ($n = 6$) *or* 1400 µl with 10 million cells/ml ($n = 6$)
Faes *et al.*	2017	20 cadaver testes from men autopsied at the department of pathology (*ex vivo*)	The average ischemia time from cardiac arrest to the time of infusion was 31 ± 17 h. (6–56 h)	RT with US– 23 gauge	50 cm HP ($n = 6$) *and* automated injection pump ($n = 14$)	^{99m}Tc-labelled GFP^{+} testicular cells isolated from mice + Microbubbles or ExEm Foam	1–2 min—50 HP *and* 0.5 ml/min for other set ups	1400 µl with 10 million cells/ml—(50 cm HP) *and* 1400 µl with 10 million cells/ml—pump *or* 2600 µl with 10 million cells/ml—pump *or* 3000 µl with 10 million cells/ml—pump

ST: seminiferous tubules, ED: efferent ducts, RT: rete testis US: ultrasonic, CT: computerized tomography, NR: not reported, GFP^{+}: green fluorescence protein, 99mtc-HMPAO: technetium-hexamethyl propylene amine oxime, HP: hydrostatic pressure

*Authors defined the injection pressure as *low*, no precise value was provided. Also, they performed the injections by hand.

Table III Characteristic of studies evaluating the effect of different volume and cell concentrations in human cadaver testis.

	Experiment set-ups	Average filled volume (mm^3)	Expansion coefficient	GFP^+ cells for seminiferous tubules (%)	GFP^+ cells for interstitium (%)	GFP^+ scope for seminiferous tubules (%)	GFP^+ scope for interstitium (%)
Faes *et al.* (2013)	800 µl with 20 million cells/ml—75 cm height	1654.6 ± 907.6[a]	2.1 ± 1.1	21.4 ± 10.1	28.6 ± 22.2	28.41 ± 33.17	–
	800 µl with 10 million cells/ml—75 cm height	2774.1 ± 1959.2	3.5 ± 2.5	21.6 ± 4.6	17.3 ± 14.6	39.59 ± 22.94	–
	1400 µl with 10 million cells/ml—75 cm height	3614.9 ± 723.1[a]	2.6 ± 0.5	24.4 ± 13.1	33.2 ± 35.7	15.32 ± 17.68	–
Faes *et al.* (2017)	1400 µl with 10 million cells/ml—50 cm height	3365 ± 1282.5	2.4 ± 0.9	4.1 ± 2.6	68.8 ± 7.9	69.6 ± 8.6	90.2 ± 4.9[f]
	1400 µl with 10 million cells/ml—500 µl/min—with AIP*	4608 ± 649.7[b, c]	3.3 ± 0.5[d, e]	11.5 ± 15.1	<%10 of the examined sections	56.5 ± 36.4	57.6 ± 44.5[f]
	2600 µl with 10 million cells/ml—500 µl/min—with AIP*	6907 ± 780.5[b]	2.7 ± 0.3[d]	1.8 ± 1.6	<%10 of the examined sections	56.0 ± 21.2	88.5 ± 5.1
	3000 µl with 10 million cells/ml—500 µl/min—with AIP*	7381.4 ± 737.4[c]	2.5 ± 0.2[e]	7.6 ± 5.5	<%10 of the each examined section	65.4 ± 43.6	89.6 ± 7.0

Only the statistical significance was shown in the Table. [a]$P = 0.01$; [b]$P = 0.019$; [c]$P = 0.010$; [d]$P = 0.019$; [e]$P = 0.005$; [f]$P = 0.020$.

Expansion coefficient (EC): based on SPECT analysis, 3D area of radioactive spot is used to describe radioactive-labelled cells and calculate their size. EC is calculated by dividing this size (mm^3) by infused volume.

GFP^+: cells positive for GFP. The percentage of GFP^+ cells was detected by immunohistochemistry as dividing the number of seminiferous tubules containing GFP^+ cells by the total number of evaluated seminiferous tubules.

GFP^+ scope for seminiferous tubules and interstitium were detected by immunohistochemistry. To obtain the scope, 'the scope of positive slides was calculated for each experiment by subtracting the depth of the positive slide at the deepest level from the depth of the slide at the lowest level.' (Faes *et al.*, 2013)

*A flow rate of 500 µl/min was provided by an automatic infusion pump (AIP).

Optimization of injection volume and cell concentration

Clinically, volume and cell concentration of the injected suspension are critical parameters for successful SSC transplantation. In an animal study, the authors demonstrated the successful regeneration of spermatogenesis in busulfan-treated adult rhesus macaque monkeys when they were injected with an average volume of 1000 µl cell suspension containing an average of 88 million viable cells per adult testis (Hermann *et al.*, 2012). Unexpectedly, pioneering studies on human testes have not initially taken into consideration the volume of fluid and concentration of cells injected. In a first attempt of *ex vivo* SSC transplantation, a total of 2 to 5 mL cell suspension and trypan blue dye were injected through the rete testis; however, the concentration of cells in the injected solution was not provided (Schlatt *et al.*, 1999). In a subsequent study, the authors injected 200 µl of dye via the rete testis; however, they did not use cells in their injection (Brook *et al.*, 2001). Although Ning *et al.* (2012) did not directly compare different injection volumes, they performed single or multiple injections (without cells) to reach a total fixed volume of 800 µl in eight human cadaver testes (Table II). Micro-CT was employed to assess the volume filled with contrast. Similar values of volume filled with contrast were obtained for the single infusion with 800 µl (2540 mm^3), the 2 × 400-µl set-up (2885 mm^3) and the 4 × 200-µl set-up (2055 mm^3) (Ning *et al.*, 2012).

In 2013, Faes and co-workers were the first to optimize volume and cell concentration conditions together for SSC transplantation in human testis (Faes *et al.*, 2013). For this, a donor cell suspension derived from adult male GFP^+ hybrid mice that had been labelled with ^{99m}technetium-hexamethylpropylene amine oxime (^{99m}Tc-HMPAO) was injected through the rete testis of 18 human cadaver testes in three different set-ups. First, an initial volume of 800 µl suspension with 20 million cells/ml was selected based on previous successful mouse transplantation trials (Ogawa *et al.*, 1997; Van Saen *et al.*, 2009). Due to the relatively low filling volumes of the first set-up, the second set was adjusted to 10 million cells/mL with the same volume. The idea behind this reduction in cell concentration was to facilitate the infusion and therefore fill more seminiferous tubules with cells. Decreasing the cell concentration led to an increased average filling efficiency. Finally, they augmented the volume to 1400 µl to increase the filling efficiency even more (Table II). Although the average filling volume was increased and showed a significant statistical difference compared to the first set-up ($P = 0.01$), the average expansion coefficient (EC; 'surface taken by a certain volume of radioactively labelled cell suspension in testis detected by SPECT') was not increased (Table III). Histological analyses of the three different set-ups revealed that the percentage of GFP^+ cells in the seminiferous tubules did not significantly differ among the groups (Table III) (Faes *et al.*, 2013).

Furthermore, in a follow-up study, 1400 µl of contrast fluid with 10 million cells/ml (isolated from GFP$^+$ hybrid mice) was infused with lower pressure in six human cadaver testes, and the authors demonstrated similar results regarding the average filling volume of the seminiferous tubules (Faes *et al*., 2017) (Table III). When they used an automatic infusion pump mimicking the 50-cm hydrostatic pressure to reduce the variability of injection, no statistical difference was found with the same volume set of 1400 µl. However, when a pump infusion with a volume of 1400 µl was compared to larger infusion volumes, statistical significance for the average filled volume was observed in higher volumes (Table III). However, the EC values of larger volumes significantly decreased when they were compared with infusions performed with a 1400 µl suspension (Table III). Moreover, although increased injected volumes resulted in a larger filled testis volume, this did not increase the amount of GFP$^+$ cells in the seminiferous tubules (Faes *et al*., 2017). As a whole, infusions with 1400 µl of 10 million cells/ml have exhibited the best filling and EC rates based on SPECT data. There seems to be little value in using higher volumes to increase the efficiency of the transplantation procedure, as the higher volumes could lead to higher leakage rates, which can hamper the success rates of transplantation. There is also a risk that higher concentrations could clog the tubules and therefore prevent transplanted cells from filling a greater amount of seminiferous tubules. A higher volume and higher concentrations could reduce the blood flow and may lead to ischemia/reperfusion injuries in clinical transplantation cases, which were not investigated in *ex vivo* human testes studies. Owing to the challenges of conducting studies in human, it is clear that we lack support for many of the inferences made from the studies described here and that further studies will be vital in evaluating SSC transplantation in human.

Deeper infusions

The extent of the tubular infusion of the injected cells is vital for the SSC transplantation efficiency. The optimal infusion would allow filling of all the seminiferous tubules with injected cells without any leakage. To perform deeper infusions without leakage, an appropriate infusion pressure and the intratesticular pressure are essential. Schlatt *et al*. (1999) were the first to associate the deeper infusion rates with small, immature or regressed testes exhibiting lower intratubular pressure (Schlatt *et al*., 1999); however, the extent of this tubular infusion was found to be inconstant, and leakage into the interstitium was observed. These results were possibly due to the different intratesticular pressures of the injected testes and high infusion pressures used by the operator. To overcome this issue, certain trials attempted to create more reliable methods. Once the infusion pressure was maintained at 75 cm constant water pressure, testes from old donors (65 to 83 years of age) were found to be prone to less tension and were more easily filled by the injection suspension (Ning *et al*., 2012). In the same study, the authors also observed infused ink particles in the interstitial tissue in seven out of eight testes, demonstrating that leaking during infusions is a prevalent issue. High-pressure levels and old-aged testes might have been the main reasons for this leakage; however, these factors were not assessed in the study (Ning *et al*., 2012).

Faes *et al*. (2013) were the first to quantify the extent to which the donor cells spread into the seminiferous tubules of human cadaver testes after SSC transplantation injection (Faes *et al*., 2013). They performed three experiment set-ups at 75 cm constant water pressure (Table III) and calculated the scope of the GFP$^+$ cells in the seminiferous tubules (the extent to which the GFP$^+$ cells spread into the seminiferous tubules) using histological examinations. Although 800 µl of the 10 million cells/mL exhibited the highest percentages of scope for GPF positivity, no significant differences were found among the experimental groups (Faes *et al*., 2013). Furthermore, leakage into the interstitium was quantified for the first time. While the lowest leakage ratio (percentage of positive cells within the interstitium) was observed for 800 µl of the 10 million cells/ml set-up, no statistically significant differences were found among the groups (Table III). Since histological analysis did not show any significant differences among the three different set-ups in terms of GFP positive cells for both the seminiferous tubules and interstitium, the data were tested for an effect of age. Testes from men older than 65 years exhibited a statistically significant difference in terms of GFP$^+$ cells in the seminiferous tubules (26.8 ± 9.0%) (mean ± SD) compared with younger testes (15.9 ± 5.3%, $P = 0.008$). Although no differences were observed for leakage into the interstitium between the old (40.8 ± 27.3%) and young testes (14.6 ± 16.8%), the old testes were more prone to leakage (Faes *et al*., 2013).

To further reduce the leakage into the interstitium, different experimental set-ups were attempted. Although decreasing the infusion pressure to 50 cm water and using an automatic infusion pump did not prevent leakage, the use of an infusion pump led to lower ratios of leakage into the interstitium (Faes *et al*., 2017) (Table III). In the clinic, automatic devices can be used to achieve deeper infusions with minimal leakage; however, further experimental studies are needed to optimize flow rate and pressure.

Limitations and future perspectives

Developing SSC transplantation to restore fertility in human males requires model systems that mimic the clinical scenario in every aspect. In the clinic, we see patients whose gonads have been insulted by toxic therapy for benign as well as malign conditions. Gonadotoxic therapy, especially the alkylating agent cyclophosphamide, and testicular irradiation deplete the germ cells (Kenney *et al*., 2001; Wallace *et al*., 2005; Jahnukainen *et al*., 2011b). Therefore, the characteristics of the testes used in these experiments have to mimic chemo- or radiotherapy-treated testes. Since human *in vivo* experiments cannot be performed for ethical reasons, experiments on human cadaver testes have been informative yet limited thus far. Overall, the limitations to SSC transplantation studies on human testes are related to the anatomical and histological features of the cadaver testes.

Leakage into the interstitium is the most common issue during the transplantation despite several attempts to lower and control the infusion pressure. Notably, this leakage may decrease the efficiency of SSC transplantation and cause some complications in live recipients. Possible factors that could lead to leakage in live recipients include fragile seminiferous tubules due to gonadotoxic therapy and intratesticular pressure. Endogenous spermatogenesis and the intratesticular blood flow are the main factors contributing to intratesticular pressure (Watson *et al*., 2015; Faes *et al*., 2017). In animal studies, the intratubular hydrostatic pressure of testis and epididymis ranges between 2 and 11 cm water pressure (Johnson and Howards 1975;

Johnson and Howards 1976). Since intratesticular pressure would be higher in the testes of living humans than those of cadavers, we may expect more resistance during transplantation, which could increase leakage. Thus, more primate studies would clarify the complex nature of pressure balance during SSC transplantation.

The long ischemia time is another drawback of the *ex vivo* studies. Since the testes were isolated from human cadavers, ischemia time, from cardiac arrest to the time of infusion, is an inevitable result and ranges from 6 to 56 h (Table II). A post-mortem study in rats demonstrated that the weight of the testes and diameter of the seminiferous tubules progressively decreased within 48 h after death. As testicular volume decreases, fewer cells can be injected (Bryant and Boekelheide 2007). The diminished diameter of the seminiferous tubules would also make the testis more prone to rupture and leakage. In the clinic, gonadotoxic therapy can also lead to shrinkage of the testis. Since live humans cannot be considered for experiments, infusion studies should be conducted on primates to address these issues.

There are several other risk factors associated with SSC transplantation. For example, rete testis injections through the skin may damage the vessels, since the rete testis is located within a highly vascular connective tissue in the mediastinum testis (Bacon and Niles 1983). Doppler ultrasonography would be useful both for avoiding blood vessels and identifying the rete testis during real transplantation cases (Belenky *et al*., 2001; Herwig *et al*., 2007).

Moreover, regardless of the transplantation technique, other factors need to be improved and clarified before translating SSC transplantation into the clinic (Fig. 3). This includes preparation of seminiferous tubules for SSC transplantation. Human cadaver studies have mostly used treatment-naive testes; therefore, the real scenario with gonadotoxic therapy was not fulfilled in these experiments. Creating sufficient space within the seminiferous tubules for exogenous germ cells is essential; therefore, the depletion of endogenous SSCs is a critical step preceding SSC transplantation (Qin *et al*., 2016a). This will allow the migration of exogenous germ cells from the lumen to basal membrane of the seminiferous tubules, an important step in SSCs inhabiting their niche for the initiation of donor-derived spermatogenesis. There are several methods for depleting endogenous SSCs successfully. The i.p. injection of busulfan, an alkylating agent, for example, has been used extensively to induce the apoptosis of germ cells and thus prepare the recipient testis (Choi *et al*., 2004; Marcon *et al*., 2011; Zohni *et al*., 2012). However, i.p. busulfan injection has been associated with haematological toxicity, which may lead to severe, and even lethal, side effects. Therefore, irradiation, another method for preparing a recipient's testis, has been developed with promising results in mouse (Zhang *et al*., 2006), rat (Abuelhija *et al*., 2013), goat (Honaramooz *et al*., 2005) and monkey (Jahnukainen *et al*., 2011a). Irradiation can be applied specifically to only the scrotum, and other adjacent organs are usually safe from radiotoxicity (Creemers *et al*., 2002); however, there is still a risk of radiation leakage, and the irradiation procedure requires special equipment, making it more expensive than other methods. There are also certain safety concerns following irradiation, since the calcification of seminiferous tubules has been reported (Zhang *et al*., 2006). This may be a barrier to a successful clinical SSC transplantation. Another method for depleting endogenous spermatogenesis is local heat-shock treatment (LHT), which has been successfully shown to destroy endogenous germ cells and block spermatogenesis in mice (Ma *et al*., 2011). However, the procedure itself is difficult to perform and, when compared with the busulfan-induced endogenous germ cell depletion method, LHT caused a smaller reduction in colony number and size, which indicates LHT's lower efficiency. The narrow transplantation window (spermatogenesis gradually recovered on day 18) (Ma *et al*., 2011) also restricts the use of LHT. To establish a safe and more feasible method, the intratesticular injection of busulfan was proposed and compared with i.p. injection of busulfan (Qin *et al*., 2016a). Following a direct injection of busulfan into the testis of mice, SSC transplantation was performed and donor-derived offspring were obtained. The cavity hollow appeared earlier in mice treated with intratesticular busulfan. Furthermore, the average luminal diameter of the seminiferous tubules gradually increased and was significantly larger compared to the i.p. busulfan-treated mice (Qin *et al*., 2016a). The testicular injection of busulfan also presented a wider window of transplantation than the LHT and irradiation methods, and its efficacy has been confirmed by several studies (Ganguli *et al*., 2016; Dong *et al*., 2019b).

A second issue that needs further clarification is the number of injected cells, which has a central role in the success of transplantation. Biopsies retrieved from prepubertal boys are limited in size and indicate a very low number of SSCs. This may necessitate *in vitro* derivation and propagation for SSC transplantation and the subsequent recolonization of seminiferous tubules (Fig. 1). Several human SSCs culturing studies have been reported (Chen *et al*., 2009; Sadri-Ardekani *et al*., 2009; Wu *et al*., 2009; He *et al*., 2010; Lim *et al*., 2010; Kokkinaki and Djourabtchi 2011; Liu *et al*., 2011; Nowroozi *et al*., 2011; Sadri-Ardekani *et al*., 2011; Mirzapour *et al*., 2012; Akhondi *et al*., 2013; Piravar 2013; Chikhovskaya *et al*., 2014; Smith *et al*., 2014; Zheng *et al*. 2014; Baert *et al*., 2015; Guo *et al*., 2015; Medrano *et al*., 2016; Gat *et al*., 2017; Dong *et al*., 2019a; Dong *et al*., 2019b). Sadri-Ardekani *et al*. were the first to report the successful propagation of human SSCs from adult and prepubertal testes; however, most of the selected molecular markers used to demonstrate the identity of human SSCs were not specific to SSCs (Sadri-Ardekani *et al*., 2009; Sadri-Ardekani *et al*., 2011). In addition, the expansion of human SSCs was not observed in subsequent studies using similar culture systems (Baert *et al*., 2015; Medrano *et al*., 2016). Other attempts to isolate and culture human spermatogonia have all been unique, and no design has been replicated in another laboratory. This non-replication is a cause for concern and suggests the need to develop a standardized protocol for the propagation of SSCs for clinical application.

Infusion of SSCs leaves a major issue of how the transplanted germ cells migrate to the basement membrane through the BTB and initiate spermatogenesis in humans (Goossens *et al*., 2013). Normally, spermatogenic cells located in the basement membrane of seminiferous tubules give rise to daughter cells that multiply and differentiate into spermatocytes that migrate off the basement membrane (Takashima and Shinohara 2018); however, in the SSC transplantation procedure, the germ cells need to migrate the opposite way to the basement membrane, and only 12% of the SSCs accomplished this in mice (Nagano *et al*., 1999). The 'homing' phenomenon is not natural; however, it underlines the local association between SSCs and their niche. Although several factors have been associated with homing efficiency, including the presence/absence of the BTB, proteins, and adhesion molecules (ITGB1, ras-related C3 botulinum toxin substrate 1, GDNF and stromal cell-derived factor 1), the homing mechanisms of SSCs are still unclear (Shinohara *et al*., 2001; Tokuda *et al*., 2007;

Kanatsu-Shinohara *et al*., 2008; Takashima *et al*., 2011; Kanatsu-Shinohara *et al*., 2012b; Dovere *et al*., 2013; Xu *et al*., 2015). The endocrine milieu also plays a role in homing efficiency, yet currently the available data support the idea that hormone suppression enhances spermatogenic recovery in SSC-transplanted primates (Shetty *et al*., 2013; Sharma *et al*., 2019). The co-transplantation of mesenchymal stem cells was also suggested to improve the homing efficiency of SSC transplantation in mice (Kadam *et al*., 2018; Kadam *et al*., 2019). However, the safety of this technique must first be established. Further studies are required to clarify the SSC–niche interaction to enhance homing efficiency.

The use of human culture systems in the clinic requires the demonstration of genetic and epigenetic stability. Goossens *et al*., (2010; Goossens *et al*., 2011) reported that there were no genetic alterations after SSC transplantation in murine; however, epigenetic alteration was observed at the stage-dependent expression of H4K5ac and H4K8ac (epigenetic modifications to the DNA packaging protein histone H4) after SSC transplantation in mice. This warrants in-depth analyses of the epigenetic changes that might influence human health.

In order to be able to translate SSC transplantation to the clinic, animal-derived culture ingredients (xenogeneic) that may transmit xenogeneic infections to humans and cause undesired immunological reactions (Chapman 2009) should be avoided. It is essential that SSC propagation should be carried out using xeno-free counterparts. As the best attempt to date to generate a xeno-free culture system for human SSCs still contained foetal bovine serum (Chen *et al*., 2009), further research is essential to develop xeno-free conditions for human SSCs (Dong *et al*., 2019b).

Although xeno-transplantation cannot be considered a real scenario owing to the aforementioned risks, allogeneic transplantation may be a useful tool for the growth of SSCs. Allogeneic transplantation of SSCs to a healthy recipient's testis with an encapsulated device that provides effective immunoprotection and presents sufficient mass transfer between the outside environment and the encased SSCs, would be an alternative way to proliferate and differentiate SSCs *in vivo*. Allogeneic transplantation has never been attempted in humans, although spermatogenesis in rhesus macaques was observed after autologous transplantation of SSCs (Hermann *et al*., 2012). The encapsulation devices were shown to be successful with parathyroid cells and a pancreatic graft in animal models; however, much progress is required for humans (Chen *et al*., 2012; Anazodo *et al*., 2019).

One of the greatest current challenges is the possibility of reintroducing malignant cells into patients (Jahnukainen *et al*., 2001). To address this issue, cell sorting methods and specific culture systems have been employed (Fujita *et al*., 2005; Sadri-Ardekani *et al*., 2014). While mouse studies were encouraging, multiparameter cell sorting methods demonstrated only partial success for human SSCs (Fujita *et al*., 2006; Geens *et al*., 2007; Dovey *et al*., 2013). Thus, further investigations are required for addressing this important issue.

Conclusion

The remarkable progress achieved in animal models is very promising for the application of SSC transplantation to restore male fertility following gonadotoxic therapies. Pretransplantation factors (SSC expansion and propagation, xeno-free conditions, endocrine milieu, etc.) have been thoroughly investigated, yet studies on human SSC transplantation are limited owing to ethical considerations. Although several human cadaver studies have endeavoured to optimize germ cell transplantation, there are still challenges to overcome. To facilitate the translation of SSC transplantation to the clinic, the optimization of cell isolation, culturing methods and SSC transplantation techniques in large animal models is necessary. Several laboratories around the world have been investigating these challenges rigorously; however, clinics should also work together to help make the goal of fertility restoration a reality for patients facing life-long infertility following lifesaving gonadotoxic therapies.

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Authors' roles

M.G.: Conception and design, Collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; L.D.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; S.H.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript, J.M.T.: provision of study material, data analysis and interpretation, final approval of manuscript; E.R.H.: manuscript writing, final approval of manuscript; C.F.S.J.: data analysis and interpretation, final approval of manuscript; J.S.: data analysis and interpretation, final approval of manuscript; D.C.: data analysis and interpretation, final approval of manuscript; J.F.: data analysis and interpretation, final approval of manuscript, C.Y.A.: conception and design, provision of study material, data analysis and interpretation, final approval of manuscript; E.G.: conception and design, provision of study material, data analysis and interpretation, manuscript writing, final approval of manuscript.

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The authors declare no competing interests.

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