Strategies for fertility preservation and restoration in the male

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Abstract

Introduction: Patients faced with infertility due to spermatogonial stem cell loss have currently semen cryobanking as only option for fertility preservation. A growing group of patients cannot benefit from this strategy as they are devoid of spermatozoa or even of any spermatogenic cell at the time of diagnosis. We therefore aimed at investigating alternative strategies to preserve or restore fertility.

Methods: As fertility preservation strategies, we investigated the reintroduction of spermatogonial stem cells by spermatogonial stem cell transplantation (SCCT) or grafting of testicular tissue pieces. To restore fertility, we explored the germ cell differentiation capacity of human embryonic stem cells (hESC). Moreover, to avoid embryo destruction during hESC derivation, we aimed to derive hESC from single blastomeres of human embryos

Results: For the SSCT, we showed that selection protocols based on magnetic and fluorescent cell sorting or selective matrix adhesion result in high germ cell-enriched fractions for transplantation. However, they are not sufficiently efficient to attain a pure germ cell fraction. After xenografting of human testicular testis tissue to immunodeficient mice, we observed long-term survival of spermatogonia within the grafts. In the fertility restoration part, we demonstrated the inductive capacity of sertoli cell-conditioned medium on germ cell differentiation from hESC. Finally, we derived two hESC from single blastomeres of two distinct four-cell stage human embryos.

Discussion and Conclusions: The fertility preservation strategies that we investigated are currently on the edge of a clinical application. In the fertility restoration path, however, more extended research will be necessary.

Key words: Spermatogonial stem cell, transplantation, fertility, hESC, differentiation, cancer, (xeno)grafting.

Introduction

Fertility has become an important issue in the concept of quality of life. At this moment, male patients faced with infertility due to the loss of spermatogonial stem cells can only be offered cryobanking of semen as a strategy to preserve their fertility. Even though semen banking is an easy and effective technique, there remains a significant group of patients (e.g. prepubertal cancer patients, Klinefelter's patients) who cannot benefit from this strategy and currently, have no option to preserve their fertility. The general aim of the work presented in this thesis was to investigate alternative strategies to help these patients to be able to father their genetically-own children in the future. The research in this thesis can be divided into two fields: on the one hand, we investigated strategies for fertility preservation using male patients' spermatogonial stem cells (SSCs), cryobanked before the onset of stem cell loss; on the other hand strategies for fertility restoration were explored for those patients facing complete SSC loss before seeking a clinical solution.

In the fertility preservation part, we studied two strategies: the (xeno)grafting of testicular tissue pieces and the transplantation of testicular cell suspensions, using the spermatogonial stem cell transplantation (SSCT) technique. As a strategy for fertility restoration, our main goal was to develop a protocol for the *in vitro* derivation of primordial germ cells and/or male gametes, starting from human embryonic stem cells (hESC). We chose hESC as a

starting point because they are the current standard for pluripotent cells. However, since the derivation of hESC from the inner cell mass (ICM) of human blastocysts implies that the donor embryo is destroyed, these cells are under heavy ethical scrutiny. Therefore, we tried to derive hESC from single blastomeres, without the destruction of the donor embryo.

1. Spermatogonial stem cell transplantation

In 1994, Brinster and Zimmerman introduced the spermatogonial stem cell transplantation (SSCT) technique (Brinster and Zimmerman, 1994). The procedure consists of the microinjection of testicular cells from a fertile male donor into the seminiferous tubules of an infertile recipient (Fig. 1). In the latter, donor-derived spermatogonia will colonize the basal compartment and donor-derived spermatogenesis will be established. In this way, the recipient male can distribute the genetic material of the germ cell donor to the next generation.

Over time, SSCT has developed into a well-established research model for the study and manipulation of SSCs. Successful transplantations were reported with both fresh and frozen-thawed cells in an increasing number of species including primates (Kanatsu-Shinohara *et al.*, 2003; Honaramooz *et al.*, 2002a; Schlatt *et al.*, 2002). The efficacy of SSCT has been demonstrated by the production of fertile offspring after spontaneous mating of transplanted animals (Brinster and Avarbock, 1994; Goossens *et al.*, 2003).

The introduction of the SSCT has opened new perspectives with regard to fertility. First of all, it represents a functional assay for male germ line stem cells and as such the SSCT has significantly increased our ability to study the fundamental biology of stem cells in the testis and male (in)fertility. Sec-

ondly, it is a potential solution for fertility preservation. Harvesting and cryostoring SSC before the start of SSC loss, and re-transplanting them into the testis of the patient after cure can theoretically result in initiation of autologous spermatogenesis, allowing the patient a "fertile future".

The SSCT technique has shown promising results in animal models with regard to fertility preservation. However, before translation to the clinic, some major concerns should be evaluated (for review: see Geens *et al.*, 2008). One of the first goals for further research is to improve the efficiency of the technique. On the one hand, enrichment of stem cells in the suspensions before transplantation might be a necessary step as the efficiency of the technique is highly correlated with the number of stem cells injected (Dobrinski *et al.*, 1999) and spermatogonial stem cells represent only a small proportion of the total testicular cells [estimated around 0.03% in mouse; in human this percentage is assumed to be higher (Tegelenbosch and de Rooij, 1993)]. On the other hand, the best method for infusion of germ cells into the human testis should be investigated (Brook *et al.*, 2001).

The safety of the technique has been mostly studied in mice. In first and second generation fetuses, born through natural conception after SSCT, all developmental parameters were comparable to controls (Goossens *et al.*, 2009). Moreover, the (epi)genetic properties of spermatozoa after SSCT were not altered (Goossens *et al.*, 2009; Goossens *et al.*, 2010), although the concentration and motility of epididymal sperm after SSCT was lower when compared to sperm from control mice (Goossens *et al.*, 2008a). Although these results are reassuring for a clinical application of the technique, more studies for safety evaluation, also in other species, will be necessary.

Also, the cryopreservation of the spermatogonial stem cells needs extra attention, as this first step will be crucial in the success of any clinical application. The first studies on cryopreservation of spermatogonial stem cells focussed on the freezing of testicular cell suspensions (Izadyar *et al.*, 2002; Hermann *et al.*, 2007). Recently, however, more attention is drawn towards the freezing of tissue pieces, as it might be important to preserve not only the spermatogonia but also the niche cells together with all the cell-cell contacts within the tissue (Kvist *et al.*, 2006; Keros *et al.*, 2007).

Last but not least, there is a risk of malignant contamination of the testicular tissue in case of cancer patients. Our research on the SSCT mainly focused on the depletion of cancer cells from contaminated testicular tissue. Hematological spread of tumors poses a significant risk for intravascular and intersti-

Fig. 1. — Set-up for spermatogonial stem cell transplantation throught the rete testis in a mouse.

tial infiltration of malignant cells in testicular tissue. In the case of acute lymphoblastic leukemia (ALL), one of the most commonly occurring malignancies in prepubertal boys, it has been shown that about 20% of newly diagnosed patients exhibit microscopic infiltration of leukaemic cells in their testes (Kim *et al.*, 1986). It is clear that reseeding contaminated testicular cells back to the patient would imply a high risk of reintroducing cancer. Therefore, the ability to remove malignant cells from testicular cells is a requisite in order to apply the SSCT technique as a clinical strategy for fertility preservation in cancer patients.

A first study aimed at evaluating the decontaminating potential of magnetic-activated cell sorting (MACS) and/or fluorescence activated cell sorting (FACS) for both murine and human testicular cell suspensions (Geens *et al.,* 2007). Artificially contaminated cell suspensions were sorted using MACS (CD49f$^+$, for mouse) and/or FACS (CD49f$^+$, H-2Kb$^-$ for mouse; HLA class I$^-$ for human) and evaluated by FACS, cell culture, transplantation and/or PCR for the B-cell receptor. In the mouse, the sorted fractions contained 0.39% H-2Kb-positive and 76.55% CD49f-positive cells. After transplantation, 1 in 20 recipient mice developed a malignancy. In the human experiments, an average of 0.58% SB cells was detected after sorting. In only 1 of 11 samples, there were no SB cells observed. These results clearly demonstrated that in this set-up, MACS and/or FACS are insufficient for completely depleting testicular tissue of malignant cells.

In a second study, we investigated whether a selective matrix adhesion-based protocol can enrich germ cells and deplete cancer cells from contaminated human testicular cell suspensions (Geens *et al.,* 2011a). Cell suspensions underwent culture selection through selective binding to uncoated dishes, collagen-I and laminin respectively. Using this protocol, an efficient enrichment of germ cells was achieved but the resulting population was not pure. Malignant cells were detected in selected cell suspensions of all patients, demonstrating that, also for this protocol, the efficiency is insufficient for clinical application.

2. Human testicular tissue grafting

An alternative technique for reintroducing cryopreserved SSCs into the testis is the intra-testicular grafting of pieces of tissue. Shinohara *et al.* (2002) showed that intratesticular grafting of donor mouse testicular tissue to an immunodeficient recipient resulted in the induction of spermatogenesis. Using sperm cells obtained from the grafts for ICSI, offspring was generated. Moreover, Van Saen *et al.*

(2009) reported that both fresh and frozen/thawed grafts showed efficient colonization of the endogenous seminiferous tubules of the recipient mouse and initiation of donor-derived spermatogenesis.

Intratesticular grafting of testis tissue has as a main advantage that the SSCs are transplanted within their original microenvironment and can be supported by donor-derived Sertoli cells. In a clinical set-up this might be important since it has been hypothesized that cancer treatment might also affect the somatic niche environment of the testis and therefore lead to an inefficient transplantation (Zhang *et al.*, 2007). Co-transplantation of SSCs within a "healthy" niche could possibly resolve this problem. However, in case of malignancy and possible malignant contamination of the testis tissue, this technique is unimaginable.

As an alternative, many research groups have thoroughly studied the ectopic (xeno)grafting of testicular tissue. Testicular tissue of different species grafted to an immunodeficient recipient mouse resulted in successful initiation of spermatogenesis with the production of fertilization-competent sperm (Honaramooz *et al.*, 2002b; Schlatt *et al.*, 2002b). After the xenografting of marmoset testicular tissue, however, a blockage at the spermatocyte stage, right before meiosis was observed (Schlatt *et al.*, 2002b; Wistuba *et al.*, 2004). Something somewhat striking, was the fact that grafting of mature testicular tissue was mostly unsuccessful, while neonatal or immature testicular grafts often resulted in complete spermatogenesis (Schlatt *et al.*, 2002b; Geens *et al.,* 2006).

Xenografting of human testicular tissue has also been under investigation (Fig. 2). In a first set of experiments, we reported the survival of human spermatogonia over a period of more than 6 months in more than 20% of adult human testicular tissue xenografts (Geens *et al.*, 2006). In a second experimental set-up, testicular tissue from two prepubertal patients suffering from severe sickle-cell anemia and in need to undergo chemotherapy and bone marrow transplantation was grafted onto the backs of six Swiss nude mice. Even at 9 months after grafting, we could still detect surviving spermatogonia (through MAGE-A4 staining) in the prepubertal testicular tissue grafts (Goossens *et al.*, 2008c). Schlatt *et al.* (2006) reported similar results on adult human testicular xenografts while Wyns *et al.* (2007) reported not only survival but also proliferation of spermatogonia in testicular tissue grafts from cryptorchid testes from young boys. However, the results from Wyns *et al.* (2007) were observed after a grafting period of only 3 weeks, and also in their experiments, no spermatogenesis was observed. Recently, our group reported meiotic activity in long-term
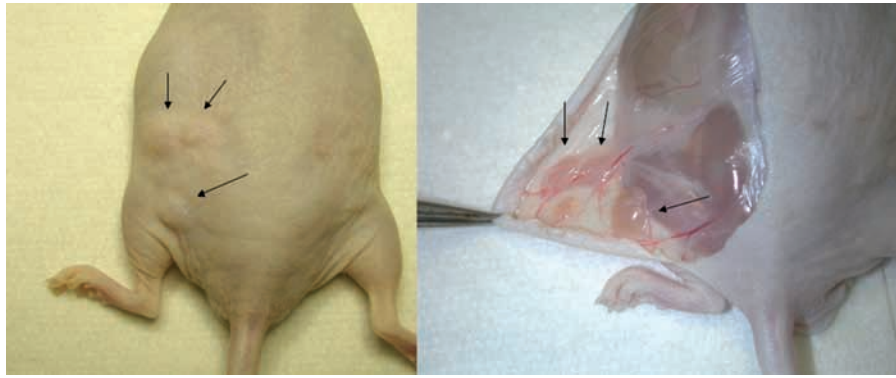
Fig. 2. — Swiss nude mouse after grafting of prepubertal mouse testicular tissue to the back. The grafted tissue (see arrows) has grown and has been vascularized by blood vessels of the host mouse.

orthotopic xenografts derived from human post-pubertal testicular tissue. However, meiosis seemed to be arrested at the spermatocyte stage (Van Saen *et al.*, 2011).

3. Fertility restoration in the absence of spermatogonial stem cells

At present, only few patients bank their SSC. Besides, the freeze and thaw protocols that are currently in use for the banking of prepubertal testicular tissue may not be optimal for the SSC because currently there is no protocol to test the functionality of the human frozen/thawed cells. At present, there is no evidence that this strategy may eventually prevent sterility on the long term as was demonstrated in mouse models. Moreover, even in the future, many patients will not have the time to cryopreserve SSC or spermatozoa before starting a gonadotoxic treatment or might not have access to a storage facility; Klinefelter's patients might already have lost all stem cells at the time of diagnosis.

A theoretical solution for those patients devoid of SSC may be fertility restoration with germ cells derived from pluripotent stem cells (e.g. embryonic stem cells, induced pluripotent stem cells). Several reasons make research on germ cell derivation from pluripotent stem cells worthwhile. First of all, hESC could serve as an *in vitro* model to study early development and more particularly germ cell determination and differentiation. It is utterly important to find an *in vitro* model for this, since *in vivo* studies of early development in human are ethically unacceptable. A better understanding of the exact processes involved in early germ cell fate determination, differentiation and maturation could open new opportunities for the treatment of infertility. Secondly, if it would be possible to derive "healthy" gametes from hESC they could also mean an enormous 'stock' of gametes for donation, solving most

of the problems of oocyte and sperm banks. In case of autologous pluripotent stem cells, it could even become possible to generate genetically own gametes for patients that became infertile due to stem cell loss or to derive 'cured' gametes from patients with genetic abnormalities before using them in ART. Moreover, *in vitro* derived oocytes could act as cytoplasmic donor in therapeutic somatic cell nuclear transfer. A third possible application of *in vitro* derived germ cells brings us back to the SSCT, where SSCs, derived from pluripotent stem cells, could be transplanted to the infertile patient, recolonize the seminiferous tubules and restore 'natural' fertility.

3A. Germ cell differentiation from pluripotent stem cells

In recent years, research on embryonic stem cell (ESC)-derived germ cells and gametes has thoroughly been studied. Germ cell differentiation from mouse ESC (mESC) has already been shown to be relatively easily reproducible as several studies reported the *in vitro* derivation of germ cell precursors or even gametes from mESC (Hübner *et al.*, 2003; Geijsen *et al.,* 2004). In 2006, Nayernia *et al.* reported the birth of 12 offspring mice, originating from ESC-derived male gametes that were used in ART. Although these results are very promising, it is important to note that the offspring mice were either smaller or larger than controls and that all of them died prematurely, presumably due to imprinting problems.

Clark *et al.* (2004) were the first group to demonstrate that human ESC (hESC) also have the potential to differentiate towards the germ cell lineage. They detected a shift in expression of early germ cell markers in undifferentiated hESC to mature germ cell markers (including *VASA*, *BOL*, *SCP1* and *SCP3)* in spontaneously differentiated hESC. Several groups tried to induce higher expression of germ cell

markers by the addition of specific growth factors known for *in vivo* germ cell induction in mouse models such retinoic acid or forskolin or by co-culturing the hESC with specific supporting cells (Richards *et al.*, 2008). Other teams focused on the selection or enrichment of germ cells in spontaneous differentiated hESC (Tilgner *et al.* 2008; Bucay *et al.*, 2009). Recently, Aflatoonian *et al.* (2009) reported the generation of haploid cells with morphology very similar to round spermatids, including the beginning of flagellum formation.

In our experiments, we aimed at investigating the germ cell differentiation capacity of VUB hESC lines by developing a protocol for the induction of germ cell differentiation using conditioned medium from Sertoli cells (SCCM), a key factor in the spermatogonial stem cell niche and comparing it to existing protocols. We found that our hESC lines are able to differentiate spontaneously into *VASA*-expressing germ cells, however, more consistently in a three-dimensional embryoid body (EB) approach than in two-dimensional monolayer cultures. Both BMPs and SCCM significantly improve VASA expression at an equal level. However, they do not have a synergistic effect. Direct contact of differentiating hESC with Sertoli cells does not further improve *VASA* expression (Geens *et al.,* 2011b). From these experiments, we concluded that SCCM contains (an) inductive factor(s) for germ cell differentiation and could therefore represent an element for *in vitro* differentiation to germ cells (Fig. 3).

3B. Human embryonic stem cell lines derived from single blastomeres of two 4-cell stage embryos.

Even though hESC are in high demand as objects of research, considerable controversies surround this issue. One of the major concerns is probably the destruction of the donor embryo, as hESC are commonly derived from the isolated inner cell mass (ICM). Hence, the derivation of pluripotent stem cells from human embryos is and will remain extremely controversial. For this reason, research is being conducted to find alternative sources of pluripotent stem cells, without the need to destroy a human embryo.

Preimplantation genetic diagnosis (PGD) has become a common procedure in fertility treatment. In standard PGD protocols, one or two cells of an 8-cell stage embryo are biopsied at day 3 after fertilization for genetic screening. The removal of one cell at this stage does not affect the vitality of the embryo (De Vos *et al.*, 2009). On the other hand, it has been shown that cryodamaged 4-cell stage embryos that had lost one blastomere developed normally (Edgar *et al.*, 2007). Therefore, it was hypothesized that single blastomeres from embryos at the 4- or 8-cell stage could be a source of cells for the derivation of ESC. Stable ESC lines have been obtained from single blastomeres of 4- and 8-cell stage mouse and human embryos (Chung *et al.*, 2005; Klimanskaya *et al.*, 2007; Feki *et al.,* 2008). These first derivations in the human however, comprised a low success rate (2%) and the necessity of co-culture with an established hESC. The protocol, however, could be improved by co-culturing the blastomere with the parent embryo and/or by adding laminin to the medium (Chung *et al.*, 2008).

Our group already demonstrated that single blastomeres of a 4-cell stage human embryo are able to develop into blastocysts with inner cell mass and trophectoderm (Van de Velde *et al.,* 2008). To further investigate potency at the 4-cell stage, we aimed to derive pluripotent human embryonic stem cells (hESC) from single blastomeres at the 4-cell stage. Therefore, four 4-cell stage embryos were split on day 2 after fertilisation and the single blastomeres were individually cultured. On day 3 or 4, the blastomere-derived aggregates were plated on inactivated mouse embryonic fibroblasts (MEFs) in standard hESC culture medium. Ten out of sixteen aggregates attached spontaneously to the MEFs, and two produced an outgrowth. The outgrowths were futher passaged and cultured as described for ICM-derived hESC and produced two hESC lines (Geens *et al.,* 2009). The two hESC lines that we derived

Fig. 3. — Immunostaining for the germ cell marker VASA in (A) human testicular tissue sections, (B) undifferentiated human embryonic stem cells and (C) human embryonic stem cells differentiated as embryoid bodies in Sertoli cell-conditioned medium.

have shown to express the typical stemness markers by immunocytochemistry and/or RT–PCR. *In vivo* pluripotency was confirmed by the presence of all three germ layers in the teratomas obtained after injection in immunodeficient mice. The first hESC line, VUB26 displays a mosaic normal/abnormal 46, XX, dup(7)(q33qter), del(18)(q23qter) karyotype. The second hESC line, VUB27 displays a normal 46, XY karyotype.

With these experiments, we succeeded in the successful derivation and characterization of two hESC lines from single blastomeres of four split 4-cell stage human embryos. These two hESC lines were derived from distinct embryos, proving that at least one of the 4-cell stage blastomeres is pluripotent. The method we described is simple and robust with an acceptably high success rate of 12.5%.

Discussion and conclusions

The presented research can be divided into two categories: on the one hand, we investigated strategies for fertility preservation using male patients' cryobanked SSCs; on the other hand strategies for fertility restoration were explored for those patients facing complete SSC loss. Both sets of strategies can potentially help patients facing possible infertility due to progressive stem cell loss. If spermatozoa are still present at the time of diagnosis, the cryopreservation of a sperm sample is a simple and very efficient means for fertility preservation. If there are no more or not yet mature spermatogenic cells, the presence of spermatogonial will determine the choice between fertility preservation and restoration strategies. If spermatogonial stem cells could be banked, they can be reintroduced to the patient's testis through tissue grafting or spermatogonial stem cell transplantation, depending on possible malignant contamination of the tissue. If there are no spermatogonia left at the time of diagnosis, germ cell differentiation from pluripotent stem cells could be a theoretical option to restore fertility (Fig. 4).

The presented fertility preservation strategies, SSCT and intratesticular tissue grafting, are very promising and eagerly awaiting the translation to the clinic. For an important group of patients, however, an important safety issue remains to be solved before any clinical application could be considered. Animal models have shown that the introduction of even a very small number of cancer cells into the testis can cause malignancy (Jahnukainen *et al*., 2001; Geens *et al.,* 2007). Due to obvious ethical reasons, it is not possible to determine the threshold number of malignant cells needed to cause malignancy relapse in the human. However, it has been assumed that a decontamination level comparable to the most sensitive detection levels used to determine minimal residual disease ($1/10^5$) in hematological malignancies should be attaint. Untill such a technique has been found, the reintroduction of autologous testicular cells cannot be applied in patients with possible malignant infiltration in the testis tissue. Clonal *in*
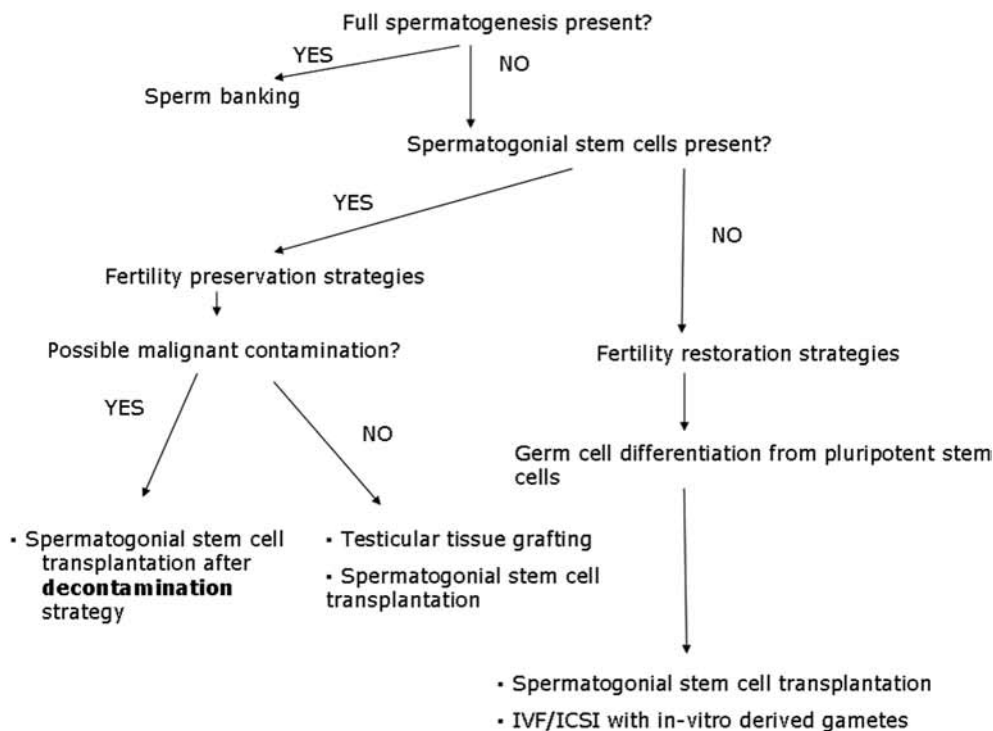

Fig. 4. — Flow chart describing the main questions regarding the choice for a possible fertility preservation or restoration strategy in case of spermatogonial stem cell loss.

vitro expansion of spermatogonial stem cells might offer a solution for malignant cell depletion (Sadri-Ardekani *et al*., 2009), however, more research in this area is still necessary.

Autologous grafting of testicular tissue to the patient could be used as an alternative method for reintroducing SSCs to the testis. Compared to SSCT, grafting of tissue is a technically easy and feasible technique (Van Saen *et al*., 2009). The grafting of testicular tissue pieces has as an important advantage that the SSCs are transplanted within the structural organization of the testicular tissue, comprising intact cell contacts and interactions that may be important for the development of the spermatogonial cells. Moreover, since it has been suggested that chemo-and/or radiotherapy may induce clinically significant impairment of the somatic elements in the testis (Zhang *et al*., 2007), it may be valuable if, in these patients, somatic cells that were not subject to a possible damaging treatment are re-transplanted together with the SSCs. As for the SSCT technique, however, the possible transfer of malignant cells remains an important disadvantage. Since it is not possible to specifically remove cells from the structure of the testicular tissue, decontamination will be impossible and therefore, this technique can only be applied in patients with non-blood cancers or in whom no metastases to the testis can occur or in patients undergoing sterilizing chemotherapy for non-malignant diseases (e.g., sickle cell anemia or thalassemia).

Many patients might be faced with infertility due to SSC loss, without any spermatogenic cell left needed for a fertility preservation strategy at the time of diagnosis. The ability of ESC and other pluripotent stem cells to give rise to germ cells in the appropriate conditions opens new perspectives for fertility restoration strategies. Gametogenesis is a process that, *in vivo*, occurs in a complex niche, supported by a delicate network of signals from the somatic environment. Whereas the *in vitro* generation of early germ cells has been reported by several groups, haploid gametes are reported less frequently. Moreover, when post-meiotic markers were detected, their expression pattern was often impaired (Novak *et al*., 2006). Meiosis is a unique functional hallmark in germ cell development. Due to the important regulatory mechanisms and specific timings that are crucial, this process is extremely difficult to be repeated *in vitro*. Therefore, it seems that only few cells are able to successfully undergo meiosis *in vitro*. Even for those few *in vitro* derived haploid gametes the functionality and safety still needs further investigation (Nayernia *et al*., 2006). Therefore, it might seem more appropriate to derive diploid SSC that can be transplanted to the testicular environment where they can proliferate, undergo meiosis and further mature

within their *in vivo* niche, supported by all necessary factors.

Even though germ cell differentiation from pluripotent stem cells could one day solve infertility by restoring fertility or by offering an unlimited source of gametes for donation, any clinical application of this technique does not seem possible for the near future. The development and improvement of differentiation protocols will need time and thorough studies of the genetic and epigenetic properties of the *in vitro* derived germ cells will be crucial. However, the study of *in vitro* germ cell differentiation could serve as an important *in vitro* model for the study of pathways involved in germ cell specification, differentiation and maturation in the early human embryo and thereby lead to new insights into germ cell commitment and differentiation, key factors in human development that are impossible to study *in vivo*. Therefore, next to the possibility of generating germ cells, the study of this differentiation process *in vitro* could help treating infertility by giving clues on the origin of the problem, hopefully making it possible to find a cure before complete fertility loss.

Fertility has become an important issue in the concept of quality of life. Our work focused on possible strategies for fertility preservation and restoration in males faced with spermatogonial stem cell loss. Although we are not able to present a straight forward protocol to help male patients facing infertility, our work has made a valuable contribution towards one or more possible solutions for patients facing infertility in the future.

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What is already known?

spermatogonial stem cells

- Spermatogonial stem cell transplantation (SSCT) has been tried with some success during the last 15 years.
- Because spermatogonial stem cells (SSCs) are scarce, enrichment of stem cells in the suspensions before transplantation is necessary in most cases.
- The best method for infusion of germ cells into the human testis remains to be investigated.
- Special consideration should be given to the risk of malignant contamination of the testicular tissue in case of cancer patients and an efficient method for cell sorting is mandatory.
- Autologous grafting of testicular tissue to the patient could be an alternative method for reintroducing SSCs to the testis. An advantage of this method is that the SSCs are transplanted within the structural organization of the testicular tissue but decontamination of malignant cells is impossible.
- Graftng can only be considered in patients with non-blood cancers or in whom no metastases to the testis can occur.

Germ cell differentiation from embryonic stem cells (hESC)

- In vitro generation of early germ cells from embryonic stem cells has been reported by several groups but haploid gametes are obtained very rarely.
- Single blastomeres of a 4-cell stage human embryo are able to develop into blastocysts with inner cell mass and trophectoderm.
- Offspring in mice has been obtained after fertilization with stem cell derived male gametes but this offsprng died prematurely, presumably due to imprinting errors.
- Meiosis in vitro is a stumbling block and genetic and epigenetic errors are to be anticipated.
- It might therefore seem more appropriate to derive diploid SSC that can be transplanted to the testicular environment where they can proliferate, undergo meiosis and further mature within their in vivo niche.

What is new from this research?

Spermatogonial stem cells

- A selection protocol based on magnetic and fluorescent cell sorting or selective matrix adhesion resulted in high germ cell-enriched fractions for transplantation but these fractions were not totally devoid of malignant cells excluding this method for cancer patients.
- Long-term survival of spermatogonia was obtained after xenografting adult human testicular testis tissue to immunodeficient mice in 20% of the grafts.
- Even more successful was the survival of spermatogonial cells after xenografting of testicular tissue from two prepubertal patients.

Germ cell differentiation from embryonic stem cells

- Two human embryonic stem cell lines were derived from single blastomeres of four split 4-cell stage human embryos. One of those cell lines had a normal 46, XY karyotype.
- Sertoli cell-conditioned medium has a positive effect on germ cell differentiation from hESC.

Which questions will these new findings arise or what problems remain to be solved?

The best procedure for transplantation of spermatogonal stem cells needs further research.

A fail-safe procedure for sorting malignant cells out of spermatogonial stem cells is mandatory.

How safe is xenografting human gametogenic tissue?

Derivation of healthy gametes from hESC for restoring fertility in man and women is the holy grail. Several conditions need to be met before this goal can be arrived at: a better understanding of the exact processes involved in early germ cell differentiation, an effective protocol for steering germ cell differentiation and last but not least, genetic and epigenetic safety.

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