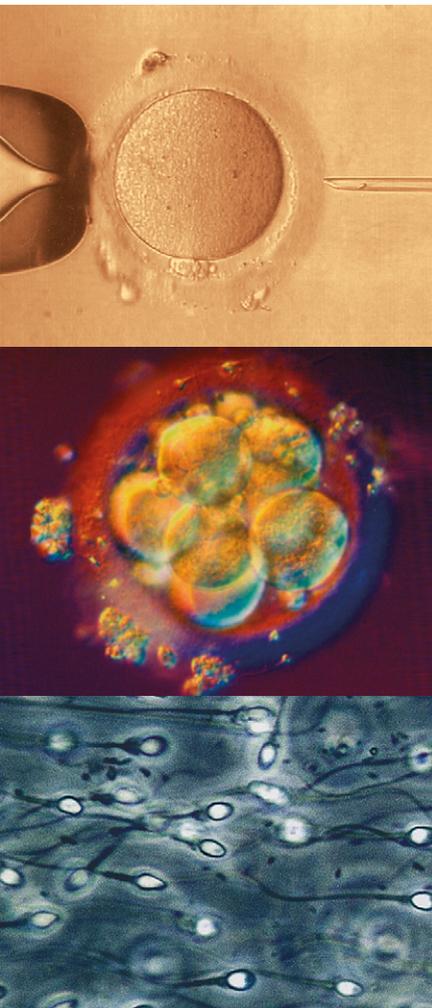Journal für

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Artificial Sperm

Nolte J, Engel W

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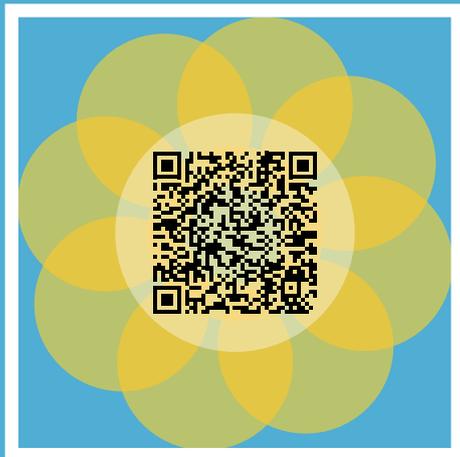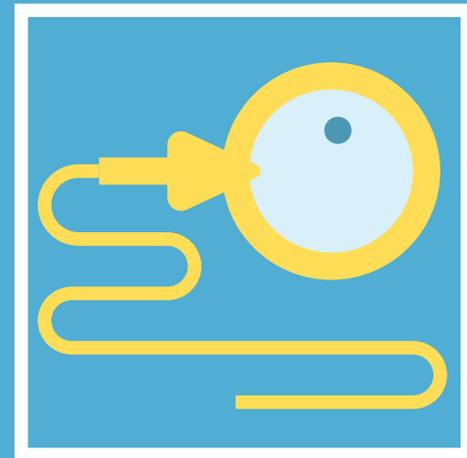
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BACK TO THE FUTURE

Artificial Sperm

J. Nolte, W. Engel

Spermatogenesis is a complex mechanism that is controlled by an extensive network of hormonal activities within an outstanding organized and structured tissue. Spermatogonial stem cells (SSCs) provide the basis of lifelong spermatogenesis by renewing the pool of SSCs balanced with differentiation into spermatogonia which will than give rise to spermatozoa. The process of spermatogenesis has been investigated by scientists all over the world for many decades.

To understand the mechanisms leading to the development of haploid gametes originating from a stem cell, is at the same time fascinating and important since this is the process that leads to the next generation. To have a tool for the investigation of this outstanding process, the establishment of robust protocols for the in vitro generation and differentiation of spermatozoa is necessary.

However, it is not only important for understanding the basics of spermatogenesis but also to create ex vivo systems for the generation of artificial sperm starting from immature germ cells from infertile patients. **J Reproduktionsmed Endokrinol 2013; 10 (Special Issue 1): 66–71**

Key words: stem cells, in vitro spermatogenesis, infertility

■ Introduction

Infertility affects 10–15% of all couples. In 50% of couples suffering from unfulfilled desire to have children, the male is the responsible factor. The main clinical phenotypes of male infertility can be summarised as spermatogenic failure and includes oligo (asthenoterato) zoospermia (OAT) and azoospermia. In most cases, the testicular phenotype of these patients consists of atrophy in the seminiferous tubuli resulting in reduced number of germ cells.

In many cases, patients show a meiotic arrest in their spermatogenesis resulting in infertility. The worst case is called Sertoli-cell-only syndrome (SCOS), where no germ cells can be found. However, the reason for the spermatogenesis defects remain unclear in about 30% of the cases and are considered as "idiopathic infertile" [1].

In mammals, lifelong spermatogenesis is sustained through stem cells in the testis. The so called Spermatogonial Stem Cells (SSCs) are set aside to the basal membrane of the seminiferous tubuli and their fraction is calculated with 0.03% of all germ cells [2]. SSCs have the potential to self renew and to differentiate to more mature germ cells and are therefore responsible for lifelong spermatogenesis in males [3–5]. SSCs emerge from primordial germ cells (PGCs) which colonize the developing testis as gonocytes before being encir-

cled by Sertoli cells. The Sertoli cells together with peritubular cells form the stem cells niche in which the SSCs are embedded. This compartment and the secreted growth factors are important for self renewal and differentiation of the SSCs. However, this stem cell niche shows a high variability between species and is still poorly understood [6]. Till now it was shown in numerous studies that the Sertoli cells secrete the growth factor GDNF (glial cell line derived neurotrophic factor). This growth factor seems to be important for the maintenance of SSCs [3, 7, 8]. In conclusion that means that the Sertoli cells are important for SSC maintenance.

To date there are no robust culture systems established for the maintenance of human SSCs in vitro. Several research groups have reported the isolation and culture of human SSCs (e.g., [9]) but no one could show the long term culture of undifferentiated SSCs. But this would be necessary to study fundamental questions about human male gametogenesis. The in vitro system has to be robust, reproducible and should result in a proper amount of cells, i.e. the SSCs should proliferate.

Another approach for the study of spermatogenesis in vitro comes from stem cell research. Stem cells are undifferentiated cells that divide continuously and have a great differentiation potential. Most reports about *in vitro* gametogenesis came from pluripotent stem cells of

different origins. Pluripotency is the ability of a stem cell to generate every cell type of the three germ layers: endoderm, mesoderm and ectoderm. Pluripotent stem cells can form any type of tissue but are not able to create a viable organism by themselves as totipotent stem cells can.

Today, several types of pluripotent stem cells are known. The gold standard was, is and will be the Embryonic Stem Cells (ESCs). First described 30 years ago [10, 11] mouse ESCs are derived from the Inner Cell Mass (ICM) of a 3.5 days *post coitum* (dpc) preimplantation embryo, the blastocyst stage. Human ESCs were first described in 1998, when Thomson et al. [12] were able to derive these cells from the ICM of human blastocysts at 5.5 dpc. All later developed pluripotent cell types had and have to be compared to ESCs to proof the potency of cell lines. Pluripotent cell types, established before ESCs are the Embryonic Carcinoma Cells (ECCs) [13]. These cells have an exceptional position as they are derived from embryonic carcinomas and not from normal cells.

Another pluripotent stem cell type are the Embryonic Germ Cells (EGCs), which can be derived from Primordial Germ Cells (PGCs), the precursors of germ cells [14, 15] at developmental stage 12.5 dpc in the mouse. The cells show all the characteristics, essential to be pluripotent. More than one decade later it was shown that those pluripotent

stem cells can not only be established from prenatal germ cells but also from neonatal germ cells. Kanatsu-Shinohara et al. [16] showed in 2004 that it is possible to derive from neonatal mice the SSCs, that can be reprogrammed just by culture conditions into pluripotent cells named mGSCs (multipotent Germline Stem Cells). This result could be enhanced by our group in 2006, when we could show the generation of pluripotent cells from SSCs isolated from adult mouse testis – in accordance to mGSCs called maGSCs (multipotent adult Germline Stem Cells) [17]. These studies and the following reassurements by other groups (e.g. [18–21]) were a big advance in reproductive biology since the idea to culture germ cells or even pluripotent stem cells derived from them gave the possibility to think about in vitro spermatogenesis based on them. To complete the list of known pluripotent stem cells: they can be isolated from post implantation embryos and are than called Epiblast Stem Cells 8 (EpiSCs) [22, 23] or be produced by reprogramming of somatic cells using defined transcription factors. These cells are than called induced Pluripotent Stem Cells (iPSCs) [24].

Several publications within the last 10 years reported about the derivation of PGCs from pluripotent stem cells of different origins. Many reports have been conducted about male germ cell differentiation till meiotic stages and some reports even about postmeiotic differentiation have been published. In the following sections we will give an overview about the different approaches starting from different cell types by shedding light on the agreements and differences and we will give a future perspective for therapeutically approaches in reproductive medicine.

Mesenchymal Stem Cells (MSCs)

MSCs are multipotent stromal cells that can be isolated as a subpopulation of bone marrow. Multipotency means that MSCs can differentiate into a limited variety of cell types [25] including osteoblasts (bone cells), chondrocytes (cartilage cells), and adipocytes (fat cells). MSCs have a large capacity for self-renewal while maintaining their multipotency. But MSCs can not differentiate

into each cell type of the body as pluripotent stem cells can. However, MSCs of mouse and human origin are shown to be able to give rise to male germ cells in vitro [26, 27]). The first study describing the in vitro generation of male germ cells from mouse MSC was conducted by Nayernia and colleagues [26]. In this study the authors used a transgenic mouse model, Stra8-EGFP. This line harbours a transgene, where the Stra8 (stimulated by retinoic acid gene 8) promoter is driving EGFP (enhanced green fluorescence protein) expression. Stra8 is a premeiotic male germ cell marker that is exclusively expressed in premeiotic male germ cell till the pachytene spermatocyte stage [28]. Isolating MSCs from bone marrow of these mice enables the later detection of premeiotic male germ cells by detection of EGFP. Induction with retinoic acid (RA) resulted in about 3% EGFP positive cells within the MSC population. The cells were then cultured under further RA treatment and analyzed after different time points.

Results indicate that mouse MSCs can differentiate to premeiotic male germ cells but fail to enter meiosis. One year later the authors describe the differentiation of human MSCs to precursors of male germ cells [27]. Just by induction with RA and using culture conditions that does not prevent differentiation, hMSCs start to express early germ cell markers like Stella, Vasa and Fragilis but also premeiotic expressed genes like Stra8, DAZL (deleted in azoospermia-like), Piwil2 (Piwi-like homolog 2) and TSPY (testis-specific protein, y-encoded).

ECCs (TCs)

Teratocarcinoma cells (TCs) can be derived from a class of non-seminomatous germ cell tumours, the teratocarcinom. It was shown that TC cell lines can be established from these tumours and that they are able to differentiate into derivatives of all three germ layers [29, 30]. Although TC cell lines have been established in vitro from transplantable testicular teratomas which contain PGCs, it is not clear whether spermatogonia can be derived from TC cells in vitro. Therefore the group of Karim Nayernia addressed this question [31]. Using the well established and described teratocarcinoma cell line F9 they developed a se-

lection strategy with which developing germ cells can be detected and isolated. Transfecting F9 cells with the Stra8-EGFP reporter construct – which was later used to generate transgenic mice [17, 27] – enabled the authors to isolate the premeiotic male germ cells via FACSorting. The cells were shown to express premeiotic marker genes. Meiotic progression of these cells in vitro could not be achieved; however, transplanting these cells into the testis of germ cell depleted mice resulted in restoration of spermatogenesis. Since the resulting spermatozoa were not motile, ICSI (intracytoplasmic sperm injection) experiments were performed, which resulted in early embryonic development.

Embryonic Germ Cells (EGCs)

Pluripotent embryonic germ cells (EGCs) can be generated by isolating primordial germ cells at fetal stage 12.5 and culturing them under appropriate conditions [14, 15]. Such cells where used by the group of Anne McLaren to generate germ cells in vitro [32]. It is known that treatment with RA can induce both, the differentiation of stem cells and the proliferation of PGCs [33–35]. The authors could demonstrate that EGCs are able to give rise to primordial germ cells under RA treatment. To further induce differentiation they used the hanging drop method combined with RA induction and a co-culture system with CHO cells. After 5–12 days of differentiation EGC derived PGCs start to express meiotic markers like SYCP3 (synaptonemal complex protein 3) proving the capacity of these cells to differentiate further.

However, it was not clearly shown, whether these cells even can form haploid germ cells and whether these cells can enter into the male or female gametogenesis.

Embryonic Stem Cells (ESCs)

Until now the largest field of in vitro generated "artificial sperm" came from embryonic stem cells research, both mouse and human. Embryonic stem cells (ESCs) are defined by their point of origin: They are derived from the inner cell mass (ICM) of a preimplanted blastocyst at day 3.5 postcoitum (dpc) in

mouse and 5.5 dpc in human. At this time the preimplanted embryo of a mouse consists of about 150 cells which form the outer layer (the trophoectoderm), a fluid-filled cavity (the blastocoel) and the ICM. To generate an ESC line, 30–40 cells of the ICM are isolated and cultured under defined conditions. ESCs – like all other pluripotent stem cells – are a suitable tool for scientists, because they combine three properties, which are not found in this combination in other cell lines: First, they are able to proliferate (also known as the possibility of "self renewal") without any differentiation or mutation of the genetic material. Second, ESCs appear genetically normal. And third, it is known that ESCs can differentiate into most cell types of an organism.

During the last decade there were several publications which figure out that ESCs also seem to be one of the most promising tools even for developmental biology and reproductive medicine: It was shown that both mouse and human ESCs were able to differentiate to primordial germ cells (PGCs), which are the precursors of both male and female gametes, and even undergo meiosis in vitro and form gametes [36–39]. These PGCs were found in embryo-like structures called "embryoid bodies" (EBs). EBs are clumps of cellular structures that arise during the culture of ESCs under culture conditions, which support the differentiation, means without the presence of leukaemia inhibitory factor (LIF) and without growing on a mouse embryonic feeder layer (MEF). The work by Toyooka et al. [38] in 2003 was the first study that demonstrates the possible differentiation of ESCs into male gametes, which show after purification, co-culture with gonadal cells and transplantation into host testis mature sperm development. Geijsen et al. [37] showed the differentiation of ESCs into more mature male gametes and these haploid gametes, when injected into oocytes, showed normal fertilisation and resulted in blastocyst formation. The study of Clark et al. [39] is the first demonstration of human ESC differentiation to germ cells in vitro. It was shown, that these cells have an indicative transcriptional profile of germ cells and mature gametes.

Despite these pioneer works about male gamete derivation from ESCs, there are numerous other studies published during the last few years (a summary is given in Table 1).

Several different approaches were used to pre-sort precursor cells of spermatogenesis and different methodical strategies where applied. The most important and functional method seems to be the formation of EBs in culture which is used in most of the reports. This can be either done in hanging drops or by culturing the cells in bacterial dishes where they can not attach. The advantage of EBs and EB-like structures seems to be on the one hand the formation of tissue like structures and on the other hand possibility for the researcher to treat the cells with different growth factors. In the work of Nayernia et al. [40], ESCs were pre-sorted for Stra8-EGFP expression (already mentioned above) and the differentiation of the cells was conducted by RA treatment under which the cells start to form EB-like structures. Within these structures, the expression of Sertoli- but not Leydig-cell markers could be detected, suggesting that within these tissue structures the niche for germ cells is built. Haploid germ cells – detectable by a Protamin1-DsRed reporter construct – seemed to migrate through these 3D structures and are released to the medium. Using these cells for ICSI experiments followed by retransfer of the resulting embryos into pseudo-pregnant host mothers resulted in viable offspring. However, mice died after different time periods due to global imprinting defects (own unpublished data). The establishment of correct imprints in the haploid male germ cells seems to be either an in vitro artefact or is dependent on the pre-sorting strategy used in the different studies.

This became more evident with the report of Hayashi et al. [41]. In this study, the authors generated viable, healthy offspring from ESC-derived PGC-like cells. Using different reporter systems and numerous of growth factors and different culture conditions, the authors first converted the stem cells to epiblast-like cells (EpiLCs) and later to PGC-like cells. Transplanting these cells into germ cell depleted mice resulted in fully restored spermatogenesis. Obviously the developed sperm was not able to fertilize oocytes naturally because the authors performed ICSI experiments with iso-lated spermatozoa. However, the resulting offspring seemed to be healthy; the mice showed normal imprinting settings and are fertile. In conclusion, the authors used a two step differentiation strategy: the first part of gamete derivation from ESCs was done in vitro and the progress through meiosis – means the production of haploid male gametes – was then achieved in vivo. However, the resulting spermatozoa arose from ESCs. Since these mice develop normal into fertile adults, the maturation in a normal environment seems to be important.

Another approach to circumvent the difficulties and obstacles that can be found in generating artificial sperm is the regulation of genes that are important for the process of spermatogenesis in vivo. Starting with mouse ESCs, Yu et al. [42] showed an efficient generation of motile tailed sperm by overexpression of the germ cell specific gene DAZL (Deleted in Azoospermia-Like). These results were supported two years later by Medrano et al. [43], who used human ESCs as well as iPSCs for their studies. By the overexpression of DAZL and/or VASA (DDX4) they achieved the efficient progress through meiosis. Analysis of maternally imprinted H19 locus revealed a positive effect especially of VASA for the re-establishment of the epigenetic imprints. Thus, a critical level of expression of important spermatogenesis specific genes seems to be necessary. Much more research is necessary to bring more details to light so that efficient generation of haploid gametes that are endowed with correct imprints can be conducted.

■ Multipotent Adult Germline Stem Cells (maGSCs)

Up to now there is only one study known which describes the generation of haploid male gem cell from maGSCs [44]. The authors used in principle the same strategy as was used before [26, 27, 40]. Shortly, the maGSCs used in this study were derived from the homozygous transgenic mouse line Stra8-EGFP and were previously shown to be pluripotent [17]. Starting from these maGSCs, the authors were able to derive premeiotic male germ cell stages through FACSorting. Interestingly it was possible to generate stable premeiotic cell lines which

Table 1: Original papers published about generation of artificial male germ cells from stem cells (this list is not exhaustive).

	Reference	Origin	Strategy for differentiation	Cell type obtained
MSCs	Nayernia et al. [26]	mouse	Stra8-EGFP transgenic mouse line; isolation of premeiotic male germ cells from MSCs by FACS; RA induction of differentiation	Premeiotic stages; arrest before entry into meiosis
	Drusenheimer et al. [27]	human	RA induction of differentiation	PGC and early germ cell marker expression; no meiotic markers
ECCs	Nayernia et al. [31]	mouse	Stra8-EGFP reporter construct; isolation of premeiotic male germ cells from F9 cells by FACS; RA induction differentiation	In vitro premeiotic stages; transplanting F9 derived SSCs in germ cell depleted testis resulted in restoration SSCs of spermatogenesis; ICSI resulted in early embryonic development
EGCs	Eguizabal et al. [32]	mouse	RA treatment combined with EB formating using hanging drop (HD) method; co-culture with CHO cells	PGCs, premeiotic germ cells, entry into meiosis (SYCP3 expression)
ESCs	Toyooka et al. [38]	mouse	Growth factors combined with EB formation	PGCs in vitro, haploide male germ cells after transplantation in vivo
	Geijsen et al. [37]	mouse	RA treatment combinded with EB formation	Haploid male germ cells (spermatids)
	Nayernia et al. [40]	mouse	Pre-selectionon SSC marker genes Stra8; RA treatment combined with EB-like formation	Haploid male germ cells; viable offspring after ICSI
	Clark et al. [39]	human	EB formation; without induction	PGCs, premeiotic germ cells; entry into meiosis
	West et al. [45]	human	Co-culture with MEFs and growth factor bFGF	PGCs, premeiotic germ cells, entry into meiosis (SYCP3 expression)
	Tilgner et al. [46]	human	EB formation; without induction	PGCs
	Bucay et al. [50]	human	Spontaneous differentiation induced by different passaging protocols	PGCs and Sertoli cells
	Aflatoonian et al. [51]	human	EB formation; without induction	PGCs; haploid male germ cells (spermatids)
	Yu et al. [42]	mouse	Overexpression of DAZL	Generation of motile tailed sperm
	Hayashi et al. [41]	mouse	Conversion of ESCs to EpiSCs and later into PGCs; transplantation into germ cell depleted testis	Fully restored spermato-genesis in vivo; normal offsprings after ICSI
	Medrano et al. [43]	human	Overexpression of DAZL and Vasa	Progression through meiosis
maGSCs	Nolte et al. [44]	mouse	Stra8-EGFP transgenic mouse line; isolation of premeiotic male germ cells from maGSCs by FACS; RA induction of differentiation	Haploid male germ cells; offsprings after ICSI could be obtained
iPSCs	Park et al. [47]	human	Co-culture with human fetal gonadal stromal cells	Early PCGs
	Imamura et al. [52]	mouse	EB formation	PGCs
	Eguizabal et al. [48]	human	RA induction followed by culture with growth factors	Progression through meiosis
	Panula et al. [53]	human	Induction with BMPs combined with the overexpression of germ cell related genes	Meiotic and postmeiotic stage
	Zhu et al. [54]	mouse	EB-like structures combined with RA induction in vitro; transplantation in germ cell depleted testis in vivo	Meiotic cells could be detected in vivo
	Yang et al. [49]	mouse	RA induction followed by co-engrafting with testicular cells	Premeiotic development in reconstituted tubules within the graft

MSCs: Mesenchymal stem sells; ECCs: Embryonic carcinoma cells; EGCs: Embryonic germ cells; ESCs: Embryonic stem cells; maGSCs: multipotent adult germline stem cells; iPSCs: induced pluripotent stem cells; Stra8: Stimulated by retinoic acid gene 8; EGFP: Enhanced green fluorescence protein; FACS: Fluorescence activated cell sorting; RA: Retinoic acid; PGCs: Primordial germ cells; F9 cells: teratocarcinoma cell line F9; SSCs: Spermatogonial stem cells; ICSI: Intracytoplasmic sperm injection; HD: Hanging drop; CHO cells: Chinese hamster ovary cells; SYCP3: Synaptonemal complex protein 3; EB: Embryoid body; EpiSCs: Epiblast stem cells; MEFs: Mouse embryonic fibroblasts; bFGF: Basic fibroblast growth factor; DAZL: Deleted in azoospermia like; BMPs: Bone morphogenetic protein

sustain the EGFP expression in about 90% of the cell population. These cells were then stably transfected with the second reporter construct, Prm1-DsRed (described in an earlier passage of this review). By withdrawal of differentiation preventing culture conditions and additional RA treatment, the cells undergo meiosis and haploid male germ cells could be detected. The process of differentiation was monitored by different molecular and epigenetic techniques proving the haploid status of the cells. Since the haploid cells were immotile and therefore unable to fertilize an oocyte by themselves, the authors performed ICSI experiments followed by retransfer of the resulting two cell stage embryos. Out of 80 embryos transferred, only two animals were born that could be clearly shown to have maGSC origin. Further studies are still needed to analyze the resulting offspring as there is no evidence given that these mice develop into normal, fertile adults that transmit the transgene (coming from the maGSCs) to the next generation.

■ Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells (iPSCs) can be generated from somatic cells by ectopic expression of four transcription factors (Oct4, Sox2, Klf4, c-Myc) in mouse and human [24, 55, 56]. Since it was shown in many comparative analyses that iPSCs have the same potential as ESCs, it is reasonable to generate germ cells from them. In a comparative approach Park et al. [47] were the first who used human ESCs and iPSCs to differentiate PGCs from them. By culturing the respective cell type on human fetal gonadal stromal cells the authors could get PGC like cells from both types supporting the similar developmental potential of ESCs and iPSCs even in the capacity to generate germ cells. Interestingly, when investigated the imprinting erasure in the presumptive PGCs, the authors found indeed the beginning of erasure but it was not complete, showing again that there might be a problem in the establishment of correct imprints in vitro. Complete meiosis from human iPSCs was firstly described by Eguizabal et al. [48]. The authors used a combination of RA induction and the addition of growth factors and inhibitors that pushed the cells through meiosis. This was also done in a comparative approach using hESCs as a control. Meiotic progression could be demonstrated by the expression of marker genes as well as analyzing the DNA content of the resulting haploid cells. Investigation of the imprinting status of the cells, however, showed again the same results: re-establishment of the paternal imprints was not complete.

One interesting paper was published very recently by Yang et al. [49]. They used a combined in vitro and in vivo approach to differentiate male germ cells from mouse iPSCs. First, they induced differentiation using RA induction. The resulting cells could be identified as premeiotic and meiotic male germ cells. These cells where mixed with testicular cells and co-engrafted under the skin of nude mice. Analysis of the grafts revealed that the iPSC-derived germ cell indeed integrated in the reconstituted tubules. However, development of the stem cell derived germ cells showed just premeiotic development. Nevertheless, the method of ectopic grafting can be a possibility to overcome the imprinting problems that can be seen in all studies published till now.

■ Outlook

The generation of artificial sperm provides an accessible way for studies of basic genetic and epigenetic mechanisms of germ cell development. But the benefit is not only for basic research and better understanding of spermatogenesis: also therapeutical approaches for the treatment of male infertility can originate from these techniques. With the breakthrough method of establishing iPSCs, we have now the tool to develop patient specific iPSCs that can be used for the in vitro spermatogenesis. Hereby we will be able to study the causes of infertility in individual patients. For example, if a patient has a block in early meiotic stages of spermatogenesis and we are able to overcome these block in vitro, one can assume that the reason for the block will be found not in the germ cell itself but in the surrounding cell types (e.g. Sertoli cells). This is on the one hand than a good chance for the particular patient to father a child through ART (Assisted Reproductive Technologies) and on the other hand interesting for the researcher to find the underlying cause in the surrounding cell type. Once the causative cell type is specified, these cells can be isolated and interesting research projects can be conducted; e.g. the expression profile of the particular cell type of the patient can be compared to healthy controls or – and this might be the more promising approach – the proteome will be analyzed by mass spectrometry. This will give clear answers whether proteins, secreted or non-secreted factors are causative for the block in spermatogenesis at the particular stage and therefore can give rise to therapeutical approaches. However, if it will not be possible to overcome the block in vitro, one can assume the underlying cause is in the germ cell itself. The most likely reason then is mutations in genes that are specifically expressed or regulated in the spermatogenic stage where the block takes place. These genes can then be found by isolation of the affected cell type followed by analyzing the transcriptome or proteome and candidate genes can be screened for mutations. Theoretically it will be even possible to correct these mutations: pluripotent stem cells can be genetically manipulated before starting the process of in vitro spermatogenesis. If indeed a mutation in a single gene is causative for the particular infertility of the patient, it should be possible to correct this mutation and consequently overcome the block in spermatogenesis in vitro.

Not only applications of clinical relevance can be performed by the establishment of proper protocols for the in vitro generation of artificial sperm but also basic research applications for the better understanding of spermatogenesis. Especially in the research of human spermatogenesis, scientists would benefit from these procedures as there will be enough material available then. For example the analysis of genetic diseases that affect germ cells or germ cell maturation and are in addition caused by genes that are not well conserved through evolution and therefore can not be studied in an animal model, will become accessible for research. Besides that, the necessity to have animal models at all for research on spermatogenesis will dramatically decrease. Only approaches that give clear and promising results in the in vitro system should then be tested in vivo through e. g. knock out studies. But even these knock-out studies can be initiated in the in vitro system

and the heterozygous spermatozoa then used for IVF or ICSI. This would additionally increase the efficiency of producing heterozygous animals compared to traditional techniques and decrease the number of animals used for such in vivo experiments.

However, a lot of work is still needed; particularly we have to solve the imprinting problems which seem to be a hallmark of successful generation of haploid male germ cells that can produce viable and healthy offspring.

■ Conflict of Interest

No potential conflict of interest to this article was reported.

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