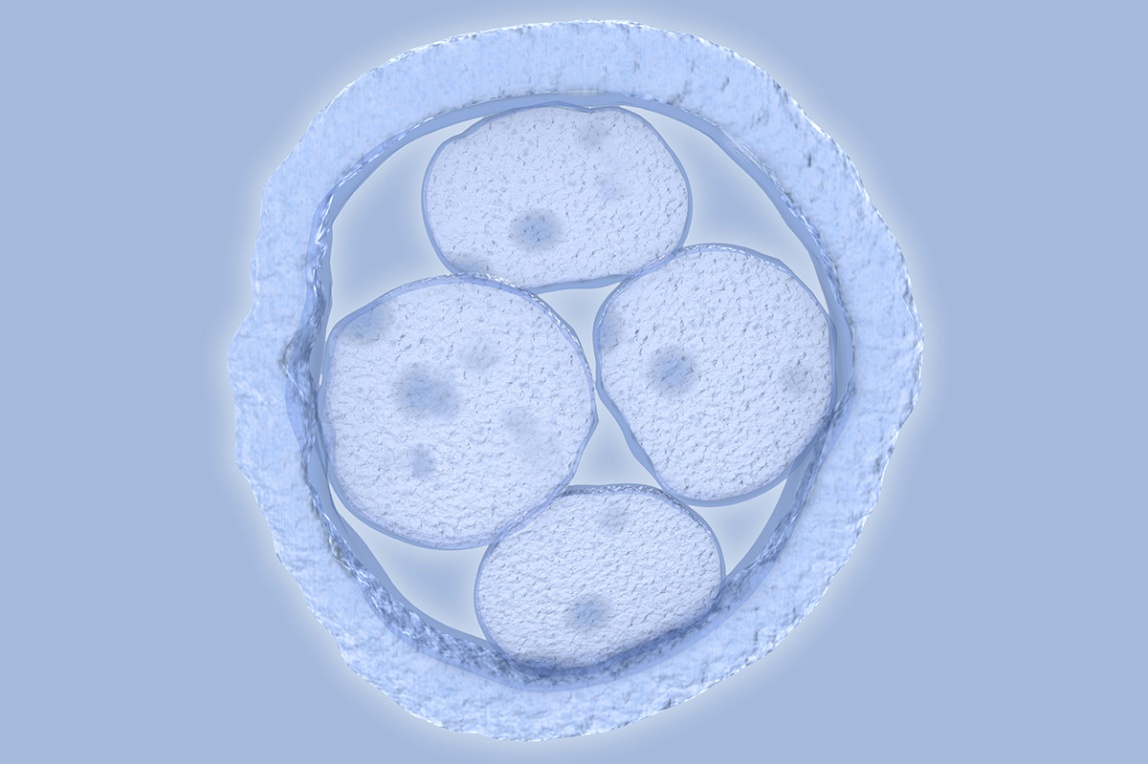
Germline Genome Editing and Induced Pluripotent Stem Cell Derived Gametes

Prospective treatment for (sub) infertile individuals or individuals with a predisposition for genetic diseases



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### Introduction

The leading causes of subfertility in men and woman are quantitative and qualitative deficits in germ-cell developments and affects 15% of couples in reproductive age (Zegers-Hochschild et al., 2009). In 17 percent of these couples a severe male factor: a total motile sperm count (TMSC) below 3x106is present (van der Steeg et al., 2007). Current assisted reproductive techniques (ART), such as IVF, ICSI, IUI. are targeted on subfertile individuals by increasing the success rate to reproduce instead of treating absolute infertile couples. It is currently impossible to treat patients without available (precursor) germ cells, for these patients donated sperm or ova is necessary. However, the offspring would not be genetically linked to either one, or both of the parents. A prospective method for this group of individuals with a wish to have genetically related progeny is to use stem cell derived gametes (SCD-gametes). SCD-gametes can be obtained out of somatic cells, for example skin cells, that are reprogrammed into induced pluripotent stem cells (iPSCs). Not only would this be an opportunity for infertile patients, it will also be a possibility for same-sex, or transgender couples to have genetically related off springs. The development of SCD-gametes do not require direct editing in the human genome. Since SCD-gametes do not alter the human genome, this method alone is not suitable to treat genetic diseases. To treat parents with a child wish who are carrying a mutant gene, a gene-editing technique is required.

Gene-editing techniques of site-specific DNA endonuclease technologies are: zinc finger nuclease (ZFN) , transcription activator-like effector nuclease (TALEN) , and the clustered regularly interspaced short palindromic repeat associated nuclease system 9 (CRISPR/Cas). These techniques offer great prospects in repair or editing in the DNA-sequences for individuals that might not be infertile but are carrying genetic inherited diseases. Both techniques combined, genome engineering applications and the SCD-gametes have the prospects of treating infertile individuals but also preventing diseases by genetic repair, without carrying out embryo selection. Although both methods have huge future advances, there are also many pitfalls. Current methods are prone to errors. Moreover editing in the human genome is by law forbidden, except for Mitochondrial DNA-transfer. Lastly, ethical arguments are also raised. This thesis explores the clinical justifications, ethical concerns, and feasibility of reproductive medicine using various germline genome editing methods and stem cell derived gametes to treat (sub) infertility or repair genetic germline mutations.

History

The first breakthroughs in reproductive medicine were made by Swammerdam, de Graaf, and van Leeuwenhoek (Repping, 2012). Since in the 17th century scientists thought living beings would originate spontaneously , Swammerdam wrote to a friend: "alhier den Almaghtigen Vinger GODS in de Anatomie van een Luys; waarin Gy wonderen op wonderen op een gestapelt sult vinden,en de Wysheid Gods in een kleen puncte klaarlyk sien ten toon gestalt”. With this statement Swammerdam laid the foundation for the reproductive biology. Soon after this, another milestone in the reproductive medicine in the 17th century was made by Antonie van Leeuwenhoek, who looked at his own sperm with his home made microscope. Van Leeuwenhoek described spermatozoa and bacteria as: ‘veele seer kleine dierkens, die daar seer aardigbeweegden’. Van Leeuwenhoek thought that the whole individual was in the sperm cell, due to the reigning theories. Van Leeuwenhoek thought that a tiny human being existed in the microscopically tiny sperm cell (Repping, 2012). He called this the 'homun-culus'. In van Leeuwenhoeks opinion, this tiny human being just needed a woman's womb to grow and nutrients. Meanwhile, Reinier de Graaf was working on studying the female and male genitalia. De Graaf, discovered a substance called the 'Graafsche follikel', which is now known under the name: antral follicle .

Although science proceeded in the 17th century, and much more was known about the reproduction, due to high risk of infection it was only since 1960 that reproductive treatments were carried out. One of these treatments involved an anti-oestrogen tablet. By admission of this tablet, woman who were not able to ovulate got a spontaneous ovulation, enabling pregnancy .The following years reproductive medicine took a big leap forwards with micro- chirurgy to open closed oviducts. However, the biggest breakthrough, was in 1978 when Steptoe and Edwards succeeded a successful pregnancy with In Vitro Fertilisation (IVF), in which fertilisation is accomplished outside the human body.

### Subfertility and infertility

Infertility can be interpret in various forms. For example, there is a distinction between infertile and subfertile couples. In first instance, the difference between both terms might seem minimal. However, the treatment for subfertile and infertile couples is completely different. For subfertile couples assisted reproductive technologies are available. These treatments increase the chance of a successful pregnancy. Subfertile couples don't have the inability to reproduce, it is a state of reduced fertility e.g. a reduced chance on meeting of oocyte and sperm due to low amounts of spermatozoa.

For infertile couples who have absence of gamete cells and precursor gamete cells (for example primordial diploid oocytes), female or male, there are no treatments yet available. All treatments at least require some kind of gamete cells, or precursor cells. However, research have been done to induce epithelial cells of infertile mice in to premordial gamete cells. This could be a prospect for stem cell embryology to treat infertile humans in the future.

# Current Assisted Reproductive Technologies

### Intrauterine insemination

Intrauterine insemination (IUI) is an assisted reproductive technique that involves the

insertion of semen in the uterine cavity. Hereby, overcoming some natural barriers

enabling sperm to ascent the female ovarian tubes. IUI does not need lab fertilization and expensive conditions such as extensive endocrine stimulation, thereby IUI is cost-effective. Moreover, IUI is a non-invasive first-line therapy for female patients with functional ovarian tubes, and subfertility due to for example: cervical factors, an ovulation, moderate male subfertility, immunological factors, and ejaculatory disorders with clinical pregnancy rates per cycle ranging from 10% to 20%. (Cohlen & te Velde, 1997).

IUI treatment with ovarian stimulation, for example with low-dose gonadotropins, significantly increases the pregnancy outcomes compared to natural cycle IUI with timed intercourse. Besides ovarian stimulation, the age of the female, the duration of sub infertility, the aetiology of the sub infertility and male factors such as: the motility of sperm and insemination count also plays important roles. An intake with a strict policy of the couple is mandatory to justify the choice of IUI, instead of other reproductive treatments. An intake in the Netherlands includes the patients (male and female) history, physical examination, and clinical and laboratory investigations. There were 58 hospitals in the Netherlands that in total carried out an IUI 19.846 times. A retrospective analyse showed that the pregnancy rate after this treatment was 9.0%, of which 7.3% conceived an offspring, see table 1 (Kremer et al. 2008). Steures et al. researched gynaecological entities in 101 Dutch hospitals, using data of the year 2003. In this research, the total started IUI-cycli, the insemination IUI-cycle, the use of ovarian stimulation, the amount of pregnancies and the amount of multiple child pregnancies. Since there are always unsuccessful pregnancies, the percentage of each variable was measured my means of a variety-analysis, taking account were the treatment took place.

|  |  |  |
| --- | --- | --- |
| Results IUI 2003 in The Netherlands | | |
| pregnancies per cycle | **Continued pregnancies per cycle** | **Multiplechild pregnancies per continued pregnancy** |
| 9,0% | 7,3% | 9,5% |

Table 1. source: Kremer et al., 2008 Tien jaar resultaten van in-vitrofertilisatie in Nederland, 1996- 2005. *Nederlands Tijdschrift Voor Geneeskunde, 152,*3, 146-52.

Subsequently, it might be more successful to treat females with endometriosis, ovarian tube blockage and/or infertility, and male infertility, who are lacking spermatozoa, with another kind of reproductive treatment. A standard procedure for an individual with open tubae is first 6 times IUI followed by 3 times IVF/ICSI.

### In Vitro Fertilization

In vitro fertilization (IVF), is retrieval of oocytes from follicles and addition of spermatozoa outside the human body. There were 9509 In Vitro Fertilization (IVF) treatments started in The Netherlands in 2015 (Table 1) (NVOG). Only 5198 embryo's were transfered (79,4 %) and in total 1290 individuals had an ongoing pregnancy. This entails a success rate of 19,08 %, This is a lot more than with IUI. However, the “burden”of treatment is much higher with IVF, because of the ovarian hyperstimulation and follicle aspiration. The first report of IVF came from Spallanzani in the 18th century, when he demonstrated the fertilization of frog oocytes by combining them with frog semen. Although he had previously seen spermatozoa using his various microscopes, at the time, it was believed that spermatozoa were parasites. In July 1978, Louise Brown was born. She was the first baby born through means of IVF developed by Steptoe and Edwards. From this moment on a true explosion took place with assisted reproductive medicine (ART) by researchers trying to optimise the methods. Whereas Louise Brown was born via natural cycles, Steptoe and Edwards also developed oocyte-stimulation methods. However, due to many failed hormonal stimulation treatments, the method was banned. After a long debate, and improving the stimulation method, of treatment of harvesting and stimulating multiple oocytes was approved. This allowed professionals to retrieve multiple oocytes within one cycle. Enabling multiple embryo's, embryo selection and embryo preservation by freezing also called cryopreservation. At first, IVF was the only option for test-tube babies. Fertilization was done by entering large numbers of spermatozoa into the test tube with oocytes. The spermatozoa had to fertilize the oocyte in a natural way, by infiltrating the cumulus and penetrating the zona pellucia. This still a natural way of oocyte fertilization, though not in a natural environment. In some cases, due to e.g. low motility of sperm cells, low sperm concentration, or abnormal shapes of spermatozoa, a fertilization by IVF was not achieved. In a latter chapter we discuss methods to treat couples with this kind of male sub-infertility with another method called Intracytoplasmic sperm injection (ICSI).

The Dutch Society for Obstetrics and Gynaecology (Nederlandse Vereniging voor

Obstetrie en Gynaecologie) achieved all year-numbers of Dutch centra where they used the In Vitro Fertilization. In 2003 the Society of National Infertility Register (Stichting Landelijke Infertiliteit Registratie) took over this role. All these annual numbers can be found at NVOG.nl. In 2015 12.6% of all cases with IVF treatment the follicle punction was stopped due to too many or too few oocytes. After a successful fertilization in 90,8% of the follicle punctions embryo transfer could take place. The chance of a pregnancy after IVF was 27,0% in 2015. Finally only 73,7% of all positive results of a pregnancy ended in a continued and successful one, due to spontaneous abortions or extra-uterine pregnancies. By analyzing all data from 1996-2005, Central Bureau for Statistics calculated that 1 in 89 children were born with the use of an IVF treatment. However, with ICSI 1 in 152 children were born. The most recent European data are from 2002. Noticeably is that the Netherlands with 1008 IVF-treatments per million citizens is lower than Scandinavian countries (2106 IVF-cycly per million citizens).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 2015 | IVF | | ICSI | | CRYO | | TOTAL | |
|  | number | % | number | % | number | % | number | % |
| Started cycles | 6509 | 100 | 7605 | 100 |  | | 14115 | 100 |
| Follicle punctions | 5687 | 87,4 | 6820 | 89,7 | 12507 | 88,6 |
| embryo transfers | 5168 | 79,4 | 6046 | 79,5 | 11327 | 100 | 22541 | 159,7 |
| Pregnancies | 1756 | 27,0 | 2181 | 28,7 | 2597 | 22,9 | 6534 | 46,3 |
| Continued pregnancies | 1290 | 19,8 | 1633 | 21,5 | 1682 | 14,9 | 4605 | 32,6 |
| Reliability interval |  | 19-21 |  | 21-22 |  | 14-16 |  | 32-33 |

Table 1: source: NVOG 2015

### Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) is a method used to treat male subinfirtility, poor quality, quantity and motility of semen are indications for ICSI. ICSI involves the insertion by microinjection of one sperm cell into the oocyte. Hereby bypassing the oocyte's defence mechanism and the fusion steps of the spermatozoon. First attempts of ICSI had been done in mice. Only 30% of injected mouse egg cells at the beginning of ICSI survived the micromanipulation procedure due to lysis of the egg cell and lysis. The succesrate for humans is higher as they have a better ratio in oocyte/spermcell ratio. By release of Phospholipase C (PLC's) prior to the sperm cell insertion, activation of the oocyte was achieved, hereby increasing the success rate of fertilization . The first successful pregnancy was obtained in rabbits by transferring the fertilized egg cells into the oviduct .The first human pregnancies from ICSI were achieved in 1992 (Palermo et al. 1992). No universal standards for patient selection have been defined. Also in the Netherlands, the main criteria to start ICSI instead of IVF is not set and differs between hospitals.

The success rate of ICSI is slightly higher than that of IVF 19,8 - 21,5 in the Netherlands (Table 1). However, this could be due to multiple variables. whilst couples who undergo ICSI have even a lower chance on natural pregnancy than IVF, one could consider doing ICSI treatments also for IVF patients. During the years ICSI treatment optimized and to a big leap in ART (table 5). IVF treatments on the other hand, are declining (table 5). In 2015 1633 children were born via ICSI of 170.510 children in total (Centraal Bureau voor Statistiek), this is 0.95 percent of the population in 2015.

### Surrogacy

Surrogacy entails fertilization of an oocyte from the biological mother by the semen of the biological father by means of IVF, afterwards the embryo is transferred to the uterus of a surrogate mother. The offspring is legally adopted by the intended parents after delivery. With other means, the child is genetically and biology linked to the biological parents, not the surrogate mother. A guideline for surrogacy in the USA was issued not earlier than 2008 (ACOG, 2008). Although it was described already in 1985 in the USA (Utian et al., 1985). The UK was the first country in Europe that started with legal surrogacy (Brinsden et al., 2000). Surrogacy was prohibited by law in the Netherlands until 1994. A gynaecological cancer patient organization raised this issue in 1986. Their aim was to make a genetic offspring legal for female who went in treatment for cancer and had were incapable of carrying their own child. Finally in 1994 the law changed, and non-commercial surrogacy became legal under strict conditions (Planningsbesluit, 1997). The change in law led to a new foundation: the Dutch Centre for Non-commercial IVF Surrogacy. Within this new organization there were several cohort studies. The main findings of the cohort study was that non-commercial IVF surrogacy is of benefit in terms of pregnancy outcome and psychological outcomes of the surrogate mother and the genetically parents of the offspring (Dermout,2001). This study was done and analyzed before the IVF treatment and after minimal one year after delivery.

Since the positive results of the study of non-commercial surrogacy, financial agreements could be made with insurance companies, which led to more IVF Surrogacy centres in The Netherlands. Hereby enabling also a solid basis for non-commercial surrogacy and enabling offspring of transgender or gay couples. All findings o the large prospective cohort study illustrated that non-commercial surrogacy is feasible, with good results in terms of pregnancy outcome and psychological outcome for the genetically linked parents and the surrogate mother, and with the absence of legal problems related to the adoption procedures.

# Current developments in infertility treatment

All fertility treatments mentioned before are focussed on increasing the likelihood of an successful pregnancy, using existing (primordial) germ cells, donor cells, or a surrogate mother. However, current developments suggest also a prospective treatment for infertile or same-sex couples using Stem Cell Derived (SCD) gametes.

Stem cell derived gametes

Recently many studies have changed the prospect on sterility and germ cell development by producing SCD gametes. In a recent study bone marrow cells were reprogrammed into pluripotent stem cells (PSC) (Shirazi et al. 2017). The stem cells express an embryonic antigen namely SSEA-1. Using magnetic-activated cell sorting (MACS) system the stem cells could be separated from the bone marrow cells. Shirazi tested the pluripotency by differentiating the stem cells towards cells of the three germ layers (ectoderm, endoderm and mesoderm). Moreover, and most important for infertility treatment the induced PSC cells were differentiated into primordial male germ cells. The expression of the transcription factors: Mvh, fragilis, DPPa3, Stra9, DAZL, indicated that the purified cells indeed showed the specific primordial germ cell markers. Thus, the results showed that SSEA-1+ pluripotent stem cells are able to differentiate into cells that express the same factors as male germ cells. Indicating that SCD-gametes may provide a genetically offspring of both parents: a male with a SCD gamete combined with a natural gamete of the female partner. Still it is very hard to derive male gametes from female cells (Nayemia et al., 2006). In short, due to the complexity of the human egg cells and s it must contain all of the resources necessary to develop into an embryo, it is very hard to derive eggs that could be used for reproduction from XY (chromosomally male) cells.Furthermore, would this procedure be technically possible, it would require extensive manipulations compared to regular gamete derivation, as a part of the male sperm cell formation information lays on the Y chromosome.

Although, induced PSC-derived gametes avoid the use of embryos, by reprogramming somatic cells, according to Easly et al, SCD-gametes are still "farm from direct clinical application". There are several obstacles such as the epigenetic stability and imprinting of SCD-gametes, therefore raising concerns of the health of the offspring. Secondly, to produce patient-specific gametes, the technique of deriving iPSCs or that of SCNT would have to be improved (Cutas et al., 2014) There is ongoing discussion about possible tumorigenicity of iPSCs as well as about increased risks for accumulation of chromosomal aneuploidies (Hendriks et al., 2015). There is also no consensus about reprogramming and validation methods to obtain iPSCs.  somatic cell nuclear transfer (SCNT), on the other hand, is regarded as a more efficient technique, although it is unclear whether SCNT-ESCs are any better than iPSCs. It has been estimated that at least in this decade SCD will not be used in clinical settings due to lack of knowledge of epigenetics regarding reprogramming cells. However, at the same time, science is focusing on the role genes in early germ cell development, the imprinting of genes and the interaction between germ cells and supporting (somatic) cells, therefore SCD still have future clinical potential.

# Current treatments for couples with a (predisposition for) genetic disease

It now has been shown what for methods currently are used to treat subfertile individuals. Moreover the prospect of SCD-derived gametes for infertile individuals or same-sex couples. However, there are also individuals who are not in/subfertile nor having a same-sex relationship that cannot or will not have children due to the fact that they are carriers of a genetic disease. In this chapter will be elaborated which current treatments currently exist for the last group mentioned.

### Preimplantation genetic Diagnosis and Preimplantation genetic Selection

Preimplantation genetic diagnosis (PGD), is the process of removing a single cell from an early stage embryo or polar body for genetic testing before transferring the embryo to the uterus. The term PGD is often misused to refer to any testing performed on an embryo prior to it being transferred to the uterus. However, there is a big difference between PGD and PGS (preimplantiation genetic screening). PGD involves collecting many cells forming multiple embryo's and testing these embryo's DNA for specific familial genetic diseases, such as cystic fibroses, before transferring a not genetic disease gene carrying embryo into the uterus. Subsequently, PGD is a kind of a priori prenatal testing. This method only test one embryo to check whether it is genetically normal or not. PGS on the other hand, is testing for overall normal chromosomes, or aneuploidy by means polymerase chain reaction (PCR) in embryos, not searching for a specific genetic diagnosis. The first reported pre-implantation genetic testing technique was reported in 1990 (Elliston, 2012). Since then major improvements took place in technology. There are multiple stages where an oocyte or embryo can be biopsied. Firstly, a polar body of the oocyte can be genetically tested. Biopsy at the cleavage stage of the embryo is done on day 3. In this period the embryo contains roughly 6-10 cells. The last type of PGD biopsy is also called trophectoderm biopsy and is performed at the blastocyst stage. Trophectoderm cells are removed out of the embryo and tested for genetic testing. PGD and PGS is relevant for a risk groups of patients. A first risk-group relevant for PGS are patients having IVF with advanced female age (~37+). This group of individuals have an increased risk of chromosomally abnormal oocytes which increases the risk of aneuploidy and thus abnormal embryos. However, research showed that this selection procedure is not reliable, as normal looking embryos could well be aneuploid or more likely mosaic - meaning different cells with a variation in genotypes. Subsequently, inserting these embryos in the uterus will lead to miscarriage or the absence of pregnancy. The Amsterdam-Groningse randomized, double blind research of 'van Mastenbroek et al.' showed that with 408 women from 35-4`years, a complement PGS to an IVF treatment, will significantly decrease the chance of a continued pregnancy. In this research chromosomes 1, 13, 16, 17, 18, 21, X and Y were screened out of one cell of an early stage embryo (4 cells or more). The cause of the decrease instead of increase of successful pregnancies could lay on multiple factors. Firstly, the FISH method is not 100% accurate, and only shows the visualisation of chromosome. Comparative genomic hybridisation (CGH) leads to a better change to diagnose aneuploidy. Secondly, removing one cell of a 4+ stage embryo could lead to damage to the early stage embryo. Lastly, and most important, it appears that mosaicism is a common phenomenon in early embryo development. This implies that not all cells in an early stage embryo have the same chromosome composition, which also happens in healthy embryos. One screened cell is therefore not representative for the whole embryo. This could lead to false-positive or negative test results. A normally healthy embryo could not be selected, and a aneuploid embryo could be screened healthy on one "healthy" cell. PGS is therefore not a reliable method, At present, the application of PGS is widely spread outside the Netherlands, but still in dispute. In some cases PGD or PGS is not relevant for genetic diseases. For instance, if the mother carries a genetic disease on her mitochondrial DNA. This part of DNA will always be passed along to the offspring. In these special cases Mitochondrial genome editing or Mitochondrial DNA transfer might be an option.

### Mitochondrial DNA transfer

Mitochondrial transfer (MT) techniques are being developed as one method of enabling

couples with a female partner who has risk on mitochondrial diseases to avoid having a child with mitochondrial disease (Richardson et al,2015). These conditions can be severe and affect multiple-organ systems. Whilst mitochondrial diseases are severe and decrease life expectancy, currently mitochondrial diseases cannot be cured. Therefore Mitochondrial DNA transfer (MT) is from great interest. There are two MT techniques: pronucleus transfer (PNT) and maternal spindle transfer (MST) (Richardson et al, 2015).MST is undertaken in oocytes and involves removing the spindle from an oocyte with genetic abnormal affected mitochondria. After removing the spindle it is inserted into an enucleated donor oocyte. After these steps ordinary IVF procedure continues. PNT involves creating an zygote using the biological gametes of the couple and transferring the pronuclei to an enucleated donorzygote.

# Current developments in genetic engineering

So far MT is the only treatment that requires genetic modifying with mitochondrial DNA in germlines and is clinically offered in a few countries. MT only involves "swabbing" parts of mitochondrial DNA, not adding or knocking out parts of Mitochondrial DNA. Moreover, it does not affect the nuclear DNA. Altering the nuclear DNA sequence is what is considered genetic engineering.

### History

In recent years researchers have successfully developed improved gene-modifying systems using alternative technologies to generate genetically engineered mammals much faster and more cost-effective compared to traditional targeted mutation methods. Although research has been done with several mammals including mice, and human cell lines, genetic engineering in nuclear DNA of humans in vivo still is speculative. Genetic engineering via homologous recombination started in 1990. From this time on, scientist could selectively knock out genes in mice, and directly evaluate the physiology, development and behaviour. Knock-out mice, generated a new platform in which different mice models would express specific disease causing genes. Therefore, it became more accessible for scientists to evaluate the characteristics of the mice without this specific genes. Moreover, researchers could replace a disease causing gene by a functional gene. This was an expensive and time-consuming process the beginning of the developments of genetic engineering. However, researchers developed technologies in which they can engineer faster, more efficient and which is less expensive. The link from mice to humans has not been founded yet. However, development is rapidly progressing, currently scientist are working on genetic modification instead of knock-out's, this involves other repairing techniques as well. In the following paragraphs recent methods and developments will be elaborated.

### Genome engineering with ZincFinger Nucleases (ZFN)

A key breakthrough in genome engineering came with the observation that the restriction enzyme FokI has molecularly distinct binding and cleavage domains, and that swapping of recognition domains could alter FokI targeting specificity. Prior to this realization, zinc fingers were discovered as a class of protein motifs in X. laevi10, and found to be frequently occurring in mammalian cells as transcription factors where bind DNA in a modular, sequences pecific manner. Each individual module of a Cys2-His2 zinc finger domain, the most commonly used ZincFinger-type (ZF) domain in genome engineering applications, contains approximately 30 amino acids that fold to interact with 3-bp of DNA. This is the domain of the Zinc Finger protein that can recognize a specific ligand, there are multiple types.

ZincFinger Nucleases (ZFNs) were the first of the genome modifying nucleases. These synthetic proteins, the natural type IIS restriction enzyme, FokI, has physically separable binding and cleavage activities (Li et al. 1992). The cleavage domain has no apparent sequence specificity. However, cutting this unspecific sequence could be replaced by substituting alternative recognition domains (Kim and Chandrasegaran 1994; Kim et al. 1996,1998). The most useful of these was a set of Cys2His2 zinc fingers (ZFs) in which approximately ~30 amino acids are bound to a Zinc atom in a conserved ββα configuration. A pair of three-finger ZFNs is shown at the top in association with a target gene in figure 1. If a homologous donor DNA is present (see the left box), Homologous-directed recombination (HDR) will occur using the donor DNA as a template. Alternatively, the break can be repaired by non homologous end joining (NHEJ), leading to mutations at the cleavage site (see right box).



Figure 1. The repairpathways of a double strand break after ZFN cleavage source: Carroll, D. (2011). Genome engineering with zinc-finger nucleases. *Genetics*, *188*(4), 773-782.

Specificity of ZFN binding is another challenge. Some fingers bind equally well to triplets other than their supposed preference, and even the best ones have some affinity for related sequences. This off-target cleavage is problematic as it will lead to cell death or even dead of the organism ((Bibikova et al. 2010; Porteus and Baltimore 2003; Alwin et al. 2005). Thus, ZFNs can be perfect in theory. However, due to many off target cleavage (lack of specifity) and the fact that double stranded breaks (DBS) can lead to mutations instead of recombination by means of NHEJ, ZFNs would not be suitable to use in vivo in humans.

### Transcription activator-like effector (TALEN)

**TALENs – Transcription activator-like effector nucleases** are similar to ZFNs since both require DNA binding motifs. ZFN and Talens cleave both at specific sites in the genome by non-specific nucleases. However, the successor: TALENs does not require re-engineering of the linkage between repeats to construct arrays, since each domain recognizes a single nucleotide instead of triplets with ZFNs, therefore it is easier for TALENS to recognize a complementary strand. Moreover, ZFN uses tri-nucleotides to target their cleavage domains, whereas Talens are less complex by using single nucleotides for DNA cleavage. Lastly, designing TALENs is generally more straightforward than ZNFs. This simplified method of modular DNA recognition by TALEN led to expansion genetic engineering (Yang et al., 2006). TALENS were first derived out of the bacteria genus Xanthomonas therefore they are natural occurring proteins. TALENS binding domain consists of 33-35 amino acid repeat domains and cleave on single base pairs (bp) (Carroll; Cathomen and Joung, 2008). TALE specificity is determined by two hypervariable amino acids also known as: repeat-variable di-residues (RVDs) (Zhang et al. 2010). Zinger-finger nucleases pioneered for almost two decades, in this time many effector domains have been made available to fuse to TALE repeats as well. Including: nucleases, transcriptional activators and site-specific recombinases (Townsend et al. 2009). The single base recognition of TALEN offers many advantages such as a greater design flexibility than the triplet-binding zinc-finger-nuclease. Although Talen is more efficient, easier to bind to the target sequence and is more flexible than ZincFinger Nuclease, it still has its limitations. For example: cloning a repeat of TALE arrays induces many challenges due to many identical repeat sequences. One way to overcome this problem is by an assembly of custom TALE arrays. By use of TALE arrays virtually any user-defined sequence can be recognized. However, TALEN still has limitations, for example: the binding site should start with a T base and still is time consuming (Zhang et al. 2010). Although Talen is an improvement on ZincFinger Nucleases, many more improvements have been made afterwards, resulting in a clustered, regularly interspaced, short, palindromic repeats associated protein-9 nuclease.

# Clustered Regularly Interspaced Short Palindromic Repeats associated protein-9 nuclease  (CRISPR-Cas9)

### introduction

The CRISPR/Cas system is one of the latest developments regarding genetic engineering technologies. CRISPR (clustered, regularly interspaced, short, palindromic repeats)/Cas (CRISPR-associated) are RNA-based systems. As with TALENS, CRISPRs mechanisms are also retrieved out of bacteria: Type II Streptococcus pyogenes. In bacteria CRISPR-Cas 9 functions by eliminating foreign DNA, which could be viruses or other invaders. These bacteria are made out of Cas endonucleases that, by use of guide-RNA (gRNA), are directed to specific sequences to cleave the targeted DNA. These gRNAs are consisting of aspecific 20 bp sequences and guides the endonuclease to a complementary target of DNA sequence. On this specific site the nuclease induces cleavage which results in a DNA double strand break (DSB). A major improvement of the CRISPR-cas system is that it won't require nuclease engineering and has less off-target effects compared to ZFN and TALENs (UL Ain et al., 2015).

The Cas9-induced DSB can be repaired either by homology-directed repair (HDR), which can occur with the presence of DNA repair templates, or by nonhomologousend joining (NHEJ) see figure 2. The error-prone NHEJ creates insertions/deletions around the point of the induced DSB. These deletions or insertions (indels) mostly occur in early coding exons and will lead to gene function loss or inducing a frame shift that results in a pre-mature stop codon (gene knockout). In contrast, HDR uses a template sequence of DNA to repair the DSB in a precise manner. These DNA repair templates can be provided to the cells together with other components of the CRISPR-Cas9 system to create specific modifications at target genomic loci. Thus, the CRISPR-Cas9 system can be used to insert sequences or correct disease-causing mutations in a very accurate way.

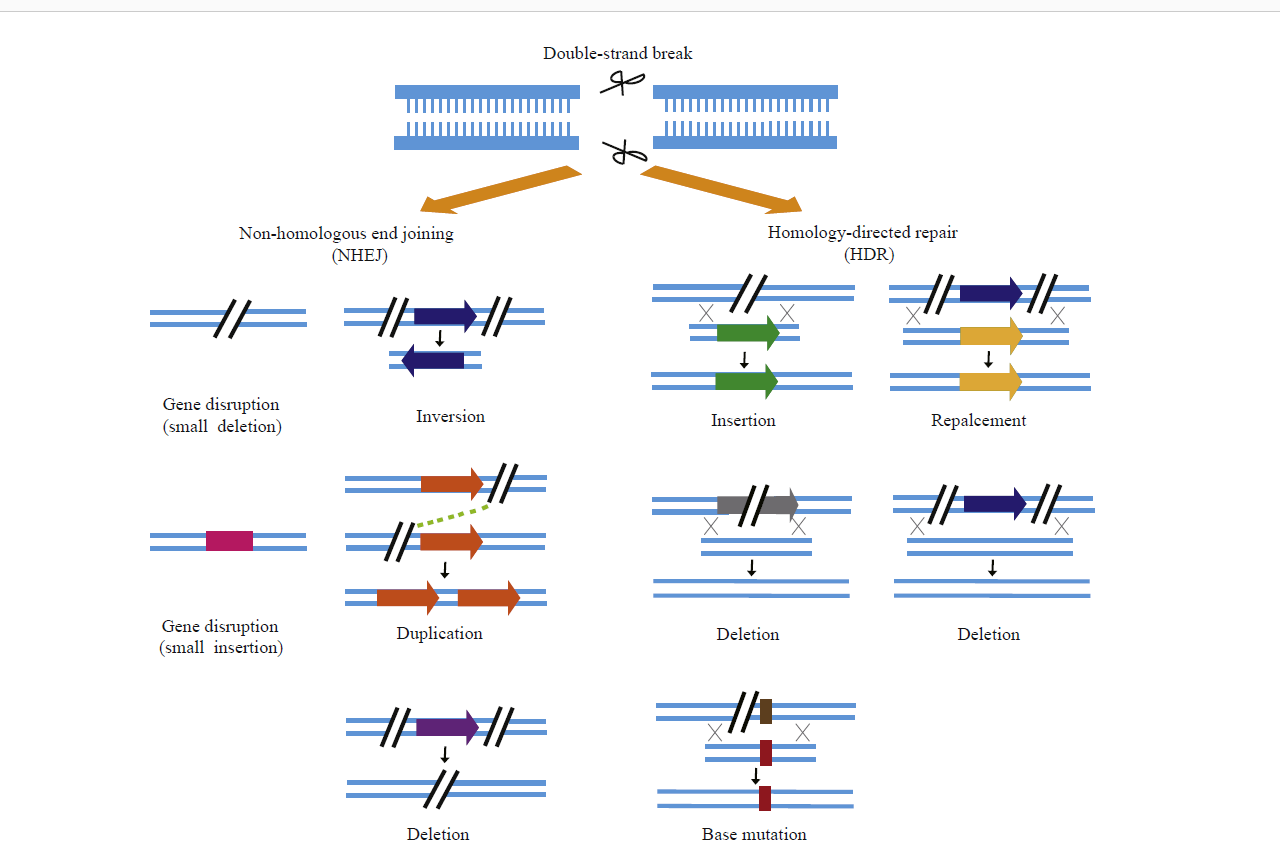


Figure 2: source: Y. Mei et al. / Journal of Genetics and Genomics 43 (2016) 63e75

There are three types of CRISPR/Cas systems. The most common system is type II which only requires a single Cas protein: Cas9. Cas 9 protein functions in RNA guided DNA recognition. To improve the CRISPR/cas9 system, variants of the type II system have been developed. Therefore enabling more and multiplexed DNA target recognition. The latest progress in the elucidation of the CRISPR/CAS modifications and methods for modifying, regulating and marking genomic loci in human cells will be elaborated.

### Development in CRISPR/Cas9 modifications

The Cas9 protein functions in nucleic acid cleavage in CRISPR/Cas systems. The crystal structure analysis revealed that the Cas9 protein contains one recognition lobe and one nuclease (NUC) lobe. The CRISPR/Cas9 has a T-shaped configuration, which is composed of the gRNA:-target sequence heteroduplex, the repeat:anti-repeat duplex, a tetraloop, three stem loops, and a linker between stem loop 1 and 2 as seen in figure 3. First, the gRNA:target sequence hetero-duplex forms when gRNA binds to the target sequence via Watson-Crick base pairing. Watson-Crick base pairing entails: hydrogen bonding patterns of guanine-cytosine and adenine-thymine for DNA, in case of RNA binding thymine will be replaced by Uracil. Second, similarly, the repeat and antirepeat regions form the repeat:anti-repeat duplex, which is necessary for Cas9 to function properly. Following, the remaining tracrRNA bases form stem loops 1, 2, and 3 via multiple Watson-Crick base pairing, respectively (Figure 3). Stem loop 1 is essential for a functional Cas9:sgRNA complex. Whereas stem loop 2 and 3 attribute to the stability and activity of the CRISPR/Cas9 complex.

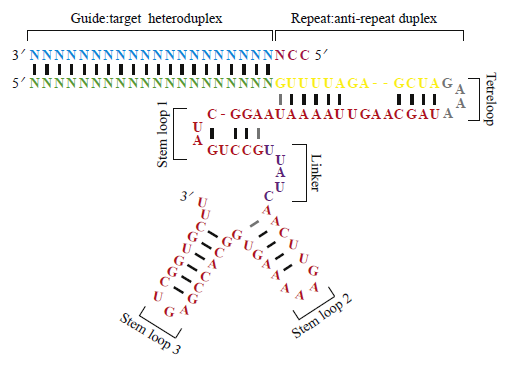


Figure 3: The overall structure of sgRNA:target DNA complex.

source: Y. Mei et al. / Journal of Genetics and Genomics 43 (2016) 63e75

Quick progress in development of CRISPR/Cas9 system is mostly due to genetic engineering of the CRISPR/Cas9 system, especially modifications of Cas9 and sgRNA. In the beginning, human codon-optimized versions of the Cas9 and RNaseIII genes, were accompanied by nuclear localization signals (NLSs) for nuclear compartmentalization to synthesize the first generation of CRISPR/Cas9 plasmids for genome editing (Cong et al.,2013). The modified Cas9 protein however, can induce a DSB at the targeted region (Fig. 4a). By generating a DSB it is activating the DNA repair mechanism to induce indels by NHEJ or HDR (Heintze et al., 2013). Due to modifications of the natural occurring Cas9, different nuclease functions arised. For example Cas9 nickase (Cas9n). Cas9n consists of an inactivating modification in the endonuclease cleavage domains (see Figure 4B and C). When Cas9n is combined with two separate sgRNAs, a staggered DSB can be produced, activating the DNA repair process, through which the efficiency and specificity of the CRISPR/Cas9 system can be further improved (Ran et al., 2013).

When Cas9 is modified such that enzymatic activities are prevented it is called Dead Cas9 (dCas9) (Wu et al., 2014). DCas9 has the characterises to target genes without modifying the DNA. This is enabled due to a blockage induced by the Cas9-sgRNA system.

The CRISPR system with a inactive enzymatic function: dCas9 is also referred to as CRISPR interference (CRISPRi) (Fig. 4D). The system of dCas9 and CRISPR offers two distinct functions. First the system can induce dCas9 fusion-mediated inhibition, also known as CRISPRi, and secondly it can induce dCas9 fusion-mediated activation (CRISPRa). Important to know is that the efficiency of both systems are depended on the gRNA sequence length (Lawhorn et al., 2014). Reaching up to 99.9% efficiency in silencing or activating multiple genes (Figure 4E). Moreover, the dCas9 system can potentially result in complex genetic re-writing, which can replace over expression or silenced of transfected plasmids in cells or organisms (Tanenbaum et al., 2014). Thus, all evidences suggest that dCas9 shows a great potential by fusion to the appropriate enzymes or transcription factors in other fields, not only in DNA modifying but also in epigenetics, cancer or neurologic disorders as it has the ability to silence or activates genes.

A new Cas9 enzyme has been developed, this is light-activated Cas9, and has great potential for spatial- and temporal-specific genome modification (see Figure 4G) (Polstein and Gersbach, 2015). This light-activated transcriptional activation CRISPR/Cas9 system contains out of two proteins. Firstly, it consist of a cryptochrome-interacting basic-helix-loop-helix 1( CIb1). This enzyme can fuse with dCas9 enabling genome targeting. Secondly, it consist of a light-sensitive crypotochrome 2 (CRY2), containing a pholotlyase region and functions as an activator probe. When both proteins, CRY2 and CIb1 are combined, the modified Cas9 will activate gene transcription and will descent to the target side. When the light-activated Cas9 activates gene transcription, it will emit blue light (Nihongaki et al., 2015). This development enables other technologies to integrate such as fluorescence microscopy and flow cytometry. This results in the opportunity to use Cas9 for screening and selection of genome-modified cells (Rojas-Fernandez et al., 2015).

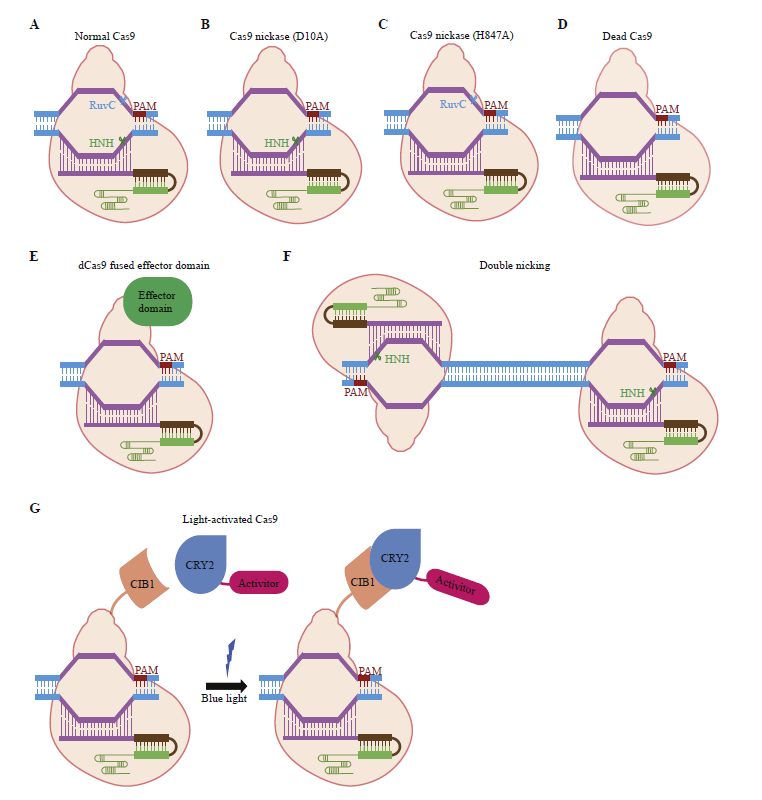


Figure 4. The modifications of Cas9 based on its functional domains. (Source: Mei et al., 2016)

With the capacity for easy and convenient genome, epigenome, and RNA editing, CRISPR/Cas9 provides immense capacity to make remarkable progress in biotechnological,

basic biological, and medical research fields. Traditional genetically modified mouse models are generated gene targeting in embryonic stem cells (ESCs). This is a time consuming and expensive method. Therefore, improving the CRISPR/Cas9 system to use it for gene function investigation or pathology would be very beneficial.

Using the CRISPR/Cas9 system to generate a mutant offspring in mice has been successful (Cong et al., 2013; Wang et al., 2013). Especially when the fertilized mice eggs were injected with Cas9 mRNa, sgRNA and a donor template in het pronuclei phase. This donor template was used to knock-in new genes and then culture the cells up until the blastocyst stage in vitro (Wang et al., 2013). Following, these blastocyst could be transfered into the mice uterus to produce a mutant offspring. This mice study has been done with mouse models who had mutations in KRAS, p53. Using CRISPR/Cas9 (Platt et al.,2014).

Not only research has been done in mice models, currently and most impressivly, CRISPR/cas9 can also disrupt infection of human immunodeficiency virus 1 (HIV-1), in human cells. This study has been done in vitro, and the replication of the virus was interrupted due to silencing or activating genes using CRISPR/Cas9. Subsequently, CRISPR/Cas9 offers great prospect for a therapeutic treatment against HIV (Liao et al., 2015)

Another virus: Hepatitis B virus, can be treated by inserting plasmids containing Cas9, mRNA, sg RNA and a donor template. Resulting in a inhibition of replication of the HBV virus. Subsequently, down-regulating the HBV protein and HBV DNA. Therefore, the CRISPR/Cas9 system may result in a new strategy for the treatment of HBV infection (Dong et al., 2015).

### Genome editing with CRISPR-CAS9 into human embryos

recently scientists used 3 or more pronuclei (3PN) human zygotes to test genomic engineering efficiency. The results were not promising with only an efficiency of 10% in HDR (Liang et al. 2015;Kang et al. 2016). Precise genome editing using the HDR pathway with a template strand is required to repair genetic defects (Heyer et al., 2010). HDR efficiency is determined generation time of the DSB site, which can be calculated. Secondly, it depends on the donor DNA concentration, and the length of the donor DNA strands (Hasty et al. 1991; Lin et al. 2014a). Fortunately, Cas9 protein is broken in pats rapidly in cells, this reduces off target insertions or deletions (Kim et al. 2014).

For this research embryos were harvested in the reproductive clinic in Guangzhou, the patients all had informed consented for the use of their 3PN zygotes. After +/-17 hours, the embryos were observed and the 3PN embryos were isolated. Cas9 and and sgRNA were added to the 3PN embryos. Afterwards the embryos were cultured for roughly 48 hours after injection. Resulting in the early morula stage consisting of 8 to 13 cells.

The genomic DNA was extracted out of the harvested embryos, amplified with a whole genome amplification (WGA) kit, and were molecular analysed. The targeted region was amplified at the target site in RAG1. These PCR-amplified products were denatured, re-annealed, and digested with T7 endonuclease I (T7E1). This endonuclease cuts as mismatches sites. In total there were 21 injected 3PN embryos. Only 12 of these injected embryos(60%) showed cleavage products, indicating a limited *RAG1* targeted editing. However, the *RAG1* targeting efficiency is comparable with the reported efficiency of 60% of the 15 observed monkey embryos for Cas9 mRNA/sgRNA injection (Niu et al. 2014). Typical indels were observed. Some embryos contained up to 3 different indels, indicating independent editing of different alleles.

Having demonstrated an efficiency of (60%) RAG1 indel creatinon by NHEJ editing. Also two other genes; G6DP and NEK1 were tested to assess the overall efficiency for NHEJ by using sgRNAs and Cas9. The observed results of this test was similar to the other genes. The *G6PD* gene infected zygotes showed about 80% efficiency and *G6PD* sand *NEK1* sgRNA were close to 70% . Subsequently, the efficiency percentage was comparable with the reported efficiencies previously in rats and hamsters: this is between 50 and 90%(Wang et al. 2013; Ma et al. 2014).

### Homologous Recombination in Human 3 or more pronuclei embryos

The NHEJ observed efficiencies of 70% of generated indel mutations were promising. Subsequently, the next step is to achieve a similar rate with a HDR method at the selected loci using Cas9 protein.As shown before, also with this method Cas9 protein is introduced to human cells via injection. Resulting in a within 3 hours induced DSB by Cas9 (Kim et al. 2014). The 3PN embryos were harvested after 16–18 hours after the insemination,

at this point the Synthesis phase (S phase) (Capmany et al. 1996). This is the phase in which embryos are in a cell cycle phase necessary for successful HDR. To test wetter Cas9-mediated HDR was efficient two genes were chosen to insert a template: the G6DP and HBB gene (B-globin). The single-straded donor template (also known as a single-strand oligo donor (ssODN), consisted of 20-50 base-pairs (Chen et al. 2011). Subsequently, for *G6PD*, we designed a ssODN of 90 nucleotides (ssODN1). This was injected into the 3PN embryos that were injected with a Cas9 protein and *G6PD* sgRNA1 at 20 hours post-insemination. Afterwards, after 2 days culturing the injected embryos were harvested for analysed. In this part of gastrulation the culture has reached the early morula stage. There were ten 3PN embryos injected. Of the ten 3PN embryos, nine showed editing at the G6DP gene. At first glance this seems promising. However, only two of these nine embryo's contained the designed template. Indicating no mutations in the other embryos. Subsequently the HDR efficiency was only 20%. Besides the second gene the *HBB*, HDR could occur at a locus much further away from the DSB generated by Cas9. To enable this research an sgRNA (*HBB* sgRNA1) was designed with a 200 nucleotide oligo donor (ssODN2). Out of the 10 3PN injected embryos, 7 showed modifications of which only one resulted from HDR, the others more likely of NHEJ. Subsequently at this locus site, the HDR efficiency was even less with 10%, which is the half of the observed 20% for injection with *G6PD* sgRNA1 and ssODN1. These results suggest that HDR efficiencies decrease some distance away from the DSB in human embryos. (Ran et al. 2013). To conclude, NHEJ efficiency with indels and point mutations are more efficient with 50-90 percent of success compared to HDR efficiency, which with 10-20 percent is not efficient. However, to correct genes which are mutated and codes for genetic diseases HDR is necessary with a template strand. To make HDR feasible in the clinic, success rates have to improve. Subsequently CRISPR/Cas methods has to improve.

To conclude, ZFNs and TALENs consist of target-specific DNA-binding domains fused to an unspecific nuclease domain. CRISPR/Cas9 to the contrary is a system with a chimeric RNA containing the guide RNA for the Cas9 nuclease to cleave the DNA (Gaj et al., 2013). The development and improvement of customized engineered endonucleases is continuously progressing but efficiencies and grade of specificity of the different nuclease systems are still controversial. TALENs and the CRISPR/Cas9 system have already replaced ZFNs as it is still technically challenging and time-consuming to engineer active ZFNs and only a few academic labs have established routine production (Maeder et al., 2008). The generation of TALENs is much less labour-intensive and time-consuming once the system has been established in the lab, although a typical TALEN requires ~1800 bp to be assembled for each new target site. The CRISPR/Cas9 system is the easiest to use, time-saving and relatively cheap, as the synthesis of an only 20 bp guide RNA is required to program the nuclease. Undoubtedly, the production of several CRISPR guide RNAs for one genomic locus and the validation of the most effective one is less time-consuming than doing the same for TALENs.

### CRISPR/Cas9 and the use of SCD gametes

Investigating hot spots for recombination sites require large numbers of oocytes. However, normally researchers can only work with small numbers of oocytes per experiment as it is impossible to retrieve large numbers of oocytes of females. The fact that researchers can only work with small numbers of egg cells comprimes the quality and efficiency of many investigations. Moreover, biochemical screening for effects of toxins and pollutants on the functionality of nondisjunction in oocytes has been limited by the quantity of available oocytes. In theory iPSCs could be used and induced into oocytes, allowing large numbers of cells to use for research purposes. iPSC technology enables oocyte formation derived out of iPSCc in specific individuals that are carriers of (un) known genetic diseases (such as: chromoso abnormalities or disease causing genes). The use of iPSCs will therefore enable experiments that are otherwise inaccessible to find the sites of mutations. Subsequently with a large number of oocytes many experiments can be done with CRISPR/Cas9. Thereby observing off-target mutations, improving the procedure in the research period. When in the future CRISPR/Cas9 has been optimized, it can also be used in clinical settings. By inducing many oocytes from the female will prevent a physically and mentally long road of invasive treatments. With many induced oocytes and the ability to check with PGD on the specific location of a known disease causing gene, cells (or embryo's when fertilized) can be selected that do not have the disease causing gene in it anymore. However, this is still a best case scenario, there are still many barriers to go before this will be safe and available in the clinical setting.

# Challenges with CRISPR/CAS9 and iPSC-derived gamete cells

Since the hypothesis is to use CRISPR-cas9 in iPSC-derived gamete cells as a prospective treatment for infertile couples or couples carrying a genetic disease, it is firstly important to see what the ethical, societal and other implications iPSC-derived gamete cells have in society and science. It could well be that in a decade the method which induces somatic cells in stem cells, and the stem cells into germ cells has improved. Including modifying epigenetic factors and ensuring genome stability.

### Challenges of the SCD gamete cells

It will be very important to have a protocol and methods than can critically assess the quality of the derived gamete cells. Till now mostly observational and biochemical tests measure the properties. However, these are inadequate to decide wetter these cells would actually develop normally. To determine fully functionality of the derived gametes out of the gamete cells, establishing their capacity for early embryogenesis is required. Moreover, and at least as important, the capacity to reproduce eventually.

Another discussion, which is more in policies not in science, is that the use PSC-derived gametes to create embryo's is not allowed in many countries, posing all kind of policy hurdles and jurisdictions. However, it has not been said that in countries where it is legal to create embryo's out of stem cells, it is also practical possible. For example, in same sex couples. Since homosexuality on its own is illegal in many countries, it is not likely that a homosexual couple could be treated by use of PSC-derived-gametes and delivering a biological child.

Besides the great prospects of induced PSC cells for reproduction, it can also be misused. For example when techniques proceeds and skin cells can be used and reprogrammed in gamete cells, a person could without consent and knowing take someone else's skin cells for their own stake and create a biological related child with the victim. To tackle this scenario strict rules and regulations have to be set up.

Moreover, one could discuss about the fact that everyone should have the right to reproduce. If so, should it also be covered in insurances for instance. That would make it unpayable for the government. However, if only the rich can afford this treatment, would that be fair or would it increase the polarisation between the rich and poor.

Another raised point is that PSC-derived gametes can be used for other purposes as reproduction. For example, with having access to the early stages of developing gametes. Scientist could observe the factors that affect the rates of chromosome nondisjunction in Meiosis I and II with oocyte development. Although this might sound still quite harmless. Some research have to been done in later stages of embryo development.

Lastly, efforts are already made to use PSC-derived gamete cells and will continue to facilitate advances biomedical sciences related to infertility and genetic disease, including chromosomal abnormalities and disease causing genes. Advantageds in the use of PSC-derived gamete cells could lead to individualised treatments for patients with these conditions. However, due to the raped progression in this field a protocol that can validate potential clinical applications is necessary to ensure safety for patients and society. This protocol should include regulations on the creation of sperm and egg cells for individuals who have lost their fertility due to disease, or for other purposes which is more controversial such as germline genetic modification, correction of disease mutations or even genetic enhancement (increase height, prevent obesity). For the this problem, which obviously requires genome genetic engineering, CRISPR-cas9 could be used. Therefore the ethical, societal and scientifical barriers will be covered in the next chapter.

### Challenges with CRISPR-Cas9

As stated before, CRISPR-Cas9 is a genetic-engineering tool that can target a particular disease causing gene in genetic disorders and repair it by inserting new genes or correcting disease causing mutations . The use of genome editing in somatic cells is already in various clinical stages and is a promising method to treat virus infection, cancers or other disorders.. CRISPR/Cas9 together with HDR could change genes will be altered and will result a change in the germline, so that also the generation after will not have the disease causing genetic gene. While CRISPR-Cas9 in germline cells could completely knock out genetic diseases, scientists have warned that it should be used with Caution (SOURCE CRISPR CAS).

### Off-target mutations

When CRISPR-cas9 cleaves on a different but homologous DNA sequence within the genome and cleave this this sequence it is called: off-target mutations. These mutations are unintended and unwanted in the genome. Multiple things could happen, the untended sequence could be spliced out, lead to cell death or cell transformation (Zhang, Wen, Guox 2014. The frequency of off-target mutations in human cells appeared much higher than with mice cells (Cryanoski, Reardon, 2015). Off-target mutations can however be decreased or avoided by using the most recent CRISPR-Cas9 developed by Yang L, et al (Yang L, Dennis G, Wang G, JohnA, Cheng-Zhong Z, et al. (2014). They increased the CRISPR-Cas9 efficiency in site-specific gene targeting using Cas9-modified hiPSC clones. However, it is still not enough to ensure no off-targets affects. This is one of the scientific arguments against using CRISPR-Cas9 in human germ cells.

### High costs of germline editing technology

The costs of editing in the germline is very high (Sourcie CRISPR CAS). Implying that only rich people could afford such treatments. Giving an advantage for developed countries. However, at the same time increasing the inequality between developing and developed countries and also within a country increasing the polarity.

### Unpredictable and unwanted applications

In the future when CRISPR-Cas9 will be used more often, it could be used for non-therapeutic modifications. For instance, humans who want aim to certain traits (e.g. increased height, different skin colour etc.) and by doing so will open the door to the loss of human diversity. For example, it was only in 2015 that researchers changed the colour of a rat skins, implying that this may be possible for humans too someday (Yang 2015). Moreover, by losing diversity in human genome can have huge impact on the resilience against (new) viruses, fungus or bacteria.

CRISPR/Cas may not be as efficient

It is not long ago that researchers thought that ZINC Finger Nuclease or TALENs would be the great breakthrough in genome engineering. However, than CRISPR/Cas9 came, based on a very different method. Why would that be not different now? Many researchers are working on optimizing CRISPR/Cas systems. But what if it almost has reached its limits. Why not focussing on another system that might be even better. If CRISPR/Cas has reached its limits now, it is certain to say it will not be used in the clinical practice of humans under the current regulations.  
Using CRISPR-Cas9 technology in embryos is risk full

Researchers are now not yet in the position to decide with precision what effects certain procedures have before birth. Since the quality control can only be performed on a subset of cells, it is not clear of the genetic modification has succeed in all cells of the embryo. Only after birth can it be figured out, but even than potential unknown problems may take years to appear (Lanphier et al., 2014).

Fate of the child

It is not clear yet what for information should be given the prospective parents to make adequately decisions. It may be clear that there will be an informed consent procedure, but it should be clearer about what the consent should be about, informing parents about the risks involved in germline modifications.

To ensure safety and well established regulations, the scientific community should discuss with stakeholders in multiple fields such as: the general public, bioethicists, public policy makers and legal experts. The previous named concerns should be implemented in the discussion enabling a well thought out plan. Regulations for human germline editing should be distinct of those of human somatic cells. So that the approaches to cure diseases will not be washed away by ethical concerns about germline genome editing.

# Conclusion: CRISPR/Cas9 and stem cell derived gametes

Concluding, in this thesis scientific backgroud, clinical justifications, ethical concerns, feasibility and social implications of reproductive medicine using germline genome editing methods and stem cell derived gametes to treat (sub) infertility or repair genetic germline mutations have been described. Currently, artificial gametes as germline genome editing are of high interest. Both methods offer great possibilities for future treatments. Not only for subfertile/infertile couples, but also for individuals with a child wish who are carrying mutant genes encoding for a disease. Especially the combination of both methods could be of high interest, using an improved CRISPR/Cas system to edit germline genome, and using induced pluripotent stem cells to differentiate into gamete cells. In this method, retrieving gamete cells of the biological parents will not be necessary anymore. However, on both methods are ethical and legal barriers and considerations. To enable these procedures, advances in technology has to be made in both methods. For genome engineering this will imply less off target mutations, and better understanding of long term consequences of germline genome editing. For the use of artificial gametes, even more developments have to be done. Including understanding and altering the methylation of DNA (epigenetics), producing viable offsprings. A modified CRISPR/CAS system could be one method to try and alter the silencing and activation of genes of artificial gametes. In some countries genetic engineering is prohibited by law, it remains important that policy makers, scientists, ethicists keep debating the pro's and cons of all methods. Future research should be done in the lab by improving genetic engineering in germ cells. But also improving and revising existing policies enabling space for researchers whilst remaining barriers to ensure biomedical safety.

# Discussion and recommendations

Until now and probably within the next decade it is clear that germline genome editing and the use of pluripotent stem cell derived gametes will not be allowed in clinical settings. However, science is progressing and there will be a time that safety the benefits will outtake the risk of any of the two mentioned methods. To make sure this period will not be chaotic, it is important to make clear regulations with a diverse group of different parties (eg. policy makers, scientists, law-experts, ethicists, socialists, doctors etc.). Moreover, prospective parents should be educated well enough to know about what they will give informed consent for. For now that is very difficult as this topic has not reached the public debate yet. Therefore not only scientist and policy makers should debate about this topic but also whole societies need to bein discussing the issues that are raised with genome engineering and pluripotent stem cell derived gametes. Although social values is important and necessary in any policy discussion, it will be important to educate parties or even societies prior to the discussion. As with the recent developed methods, it might be even more important for researchers to maintain an open view and think further, who knows a total different method will have even more benefits.

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GLOSSARY

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| TALENs | Transcription activator-like effector (TALE) nucleases are fusions of the *Fok*I cleavage domain and DNA-binding domains derived from TALE proteins. TALEs contain multiple 33–35 amino acid repeat domains that each recognizes a single base pair. TALENs induce targeted DSBs that activate DNA damage response pathways and enable custom alterations. |
| CRISPR/Cas (CRISPR associated) systems | Clustered Regulatory Interspaced Short Palindromic Repeats or CRISPR are loci that contain multiple short direct repeats, and provide acquired immunity to bacteria and archaea. CRISPR systems rely on crRNA and tracrRNA for sequence-specific silencing of invading foreign DNA. Three types of CRISPR/Cas systems exist: In type II systems, Cas9 serves as an RNA-guided DNA endonuclease that cleaves DNA upon crRNA-tracrRNA target recognition. |
| DSB | Double-stranded breaks are a form of DNA damage that occurs when both DNA strands are cleaved |
| NHEJ | Non-homologous end joining is a DSB repair pathway that ligates or joins two broken ends together. NHEJ does not use a homologous template for repair and thus typically leads to the introduction of small insertions and deletions at the site of the break, often inducing frame-shifts that knockout gene function. |
| HDR | Homology-directed repair is a template-dependent pathway for DSB repair. By supplying a homology-containing donor template along with a site-specific nuclease, HDR faithfully inserts the donor molecule at the targeted locus. This approach enables the insertion of single or multiple transgenes, as well as single nucleotide substitutions. |
| RNAi | RNA interference is the process by which RNA molecules inhibit or knockdown gene expression. More broadly, RNAi is a natural mechanism that occurs in response to the introduction of many types of RNA molecules into cells. |
| ZFNickases | Zinc-finger nickases are ZFNs that contain inactivating mutations in one of the two *Fok*I cleavage domains. ZFNickases make only single-strand DNA breaks and induce HDR without activating the mutagenic NHEJ pathway. |