Part A – Applicant

A.1 Applicant

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Part B – Scientific proposal

B.1 BASIC DETAILS

B.1.1 Title

Derivation of functional oocytes from frozen pediatric ovaries of childhood cancer patients

B.1.2 Abstract

Cancer therapies for pediatric patients may induce sterility. Therefore, it is necessary to develop fertility preservation strategies to allow survivors of pediatric cancer to produce offspring later in life. For female pediatric cancer patients, one such strategy is the removal of ovaries or the freezing of oocytes before treatment, followed by later reimplantation of the ovaries or IVF using the cryopreserved oocytes. However, this is currently not feasible in pediatric patients whose ovaries have not yet undergone puberty. A part of the problem is formed by the difficulty of maturing pediatric oocytes in vitro. We present a proposal for a protocol that can be used to culture and develop primordial follicles derived from cryopreserved pediatric ovaries to obtain oocytes that can be successfully fertilized in vitro. Following follicle culture with Activin A and FSH to obtain antral follicles, in vitro maturation (IVM) will be attempted using specific IVM media combined with hormones and factors that enhance IVM efficiency such as HCG and LH. Alternatively, it may be possible to aggregate adult ovarian granulosa cells together with pediatric primordial follicles as a strategy of developing primordial follicles in vitro. This will pave the way for clinical application of IVM to preserve the fertility of pediatric cancer survivors, a currently unmet medical need. Additionally, our study may lead to an increased understanding of human gamete development and aid efforts to generate mature functional oocytes in vitro from human induced pluripotent stem cells (iPSCs), allowing advanced fertility therapies.

B.1.3 Layman's summary

Cancer patients may have to undergo specific chemotherapeutic treatments which can damage their reproductive organs, leading to infertility. For such patients, it may be beneficial to remove parts of their gonads and freeze them in order to reimplant them later after the cancer therapy has ended. For example, women can have their ovaries frozen, or oocytes can be extracted from the ovaries and then frozen and stored to be used later with IVF. This is problematic in young female cancer patients whose ovaries have not yet undergone puberty because their ovaries do not produce ovulatory follicles yet and there are no mature, competent oocytes to vitrify. Recently, advancements have been made in the in vitro follicle culture of human oocytes. This is the process where immature follicles are cultured in special media in the presence of specific hormones outside of the body, eventually allowing the development of a mature, fertile oocyte from an immature follicle after IVM of the antral follicle. This has already been well described in mice, where fertile offspring have been produced from oocytes generated

from immature follicles and even from mouse embryonic stem cells. However, the process is difficult to recapitulate in vitro with human oocytes.
Combining the currently available knowledge, we will develop and refine a protocol to first culture primordial follicles which are present in pediatric ovaries, and then mature the oocytes in vitro to a state that resembles the oocytes found in vivo following ovulation. For this purpose, we will be working with frozen material obtained from young female cancer patients. We will use hormones to stimulate the primordial follicles to grow, after which we will apply pressure to obtain the oocyte and its surrounding cells from the cultured follicle. We will then culture the oocyte in a special medium together with different combinations of hormones to evaluate which combination works the best. Alternatively, we will isolate the hormone producing cells from adult human ovaria and culture them together with primordial follicles. This will simulate the in vivo environment of adult human ovaries, thereby stimulating the primordial follicles obtained from the pediatric patients to grow and become ovulatory.
The information obtained will have paved the way for fertility preservation of female pediatric cancer survivors, which is a currently unmet medical need. Our research will also significantly contribute to this research field, enhancing our understanding of the way that immature human oocytes develop into fertile reproductive cells. This can in turn lead to further advances, enabling us to provide novel fertility therapies for infertile persons.

B.1.4 Keywords

Ovarian tissue cryopreservation
In vitro oocyte maturation
Fertility preservation
Cancer

B.2 SCIENTIFIC PROPOSAL

B.2.1 Research topic

Pediatric cancer, defined as cancer that occurs between birth and 18 years of age, is a devastating condition that affects hundreds of children in the Netherlands each year (1). The treatment of pediatric malignancies is particularly problematic for female patients, as chemotherapy can be severely gonadotoxic in females and can induce sterility (2). The severity and specific consequences of chemotherapy-induced gonadotoxicity vary by treatment regimen, duration, and the length of exposure. One of the key risks of chemotherapy in female pediatric cancer patients is premature ovarian insufficiency (POI) (3). This condition arises when the pool of ovarian follicles is depleted or dysfunctional before the age of 40, leading to an early loss of fertility in women.

To preserve fertility, ovaria can be excised and cryopreserved in liquid nitrogen, or oocytes can be extracted and vitrified. In adult patients, this provides a pool of ovarian follicles which can later be used for in vitro fertilization (IVF), thereby preserving the fertility of the patient. Alternatively, it is possible to reimplant the cryopreserved ovaries into the patients following the cancer treatment. This does, however, carry the risk of reintroducing malignant cells, necessitating close histological analysis of the reintroduced tissue (4). Unfortunately, neither approach is feasible for pediatric cancer patients whose ovaries have not yet fully developed at the time of excision. Reimplantation procedures with such patients have not been successful (4), and the immature ovaria do not possess mature oocytes which can be extracted, vitrified, and used for IVF.

Thus, fertility preservation for female pediatric cancer patients is currently an unmet medical need. Research efforts in this area have focused on unravelling the complex maturation pathway that oocytes undergo in vivo and then replicating this pathway in vitro using recombinant hormones as well as advanced artificial extracellular matrix media (5–7).

The developmental process of an oocyte is complex and consists of several phases. The first precursors of the ovaries and oocytes arise during very early stages of development in the epiblast, where a population of cells differentiates into primordial germ cells (8). These primordial germ cells remain indifferent at first until sex specific signalling occurs whereupon the development of the ovaries initiates. During this time, the oocyte undergoes an epigenetic reset as DNA methylation is erased, and various germ cell specific markers such as BLIMP1 and VASA are expressed (7). The recapitulation of complete oocyte development in vitro is a daunting task which involves manipulation of multiple developmental pathways. Rather than inducing expression of these factors through genome editing, it is much more attractive to trigger and maintain this pathway through the application of outside factors such as ovulation and pregnancy related hormones. Not only does this method avoid disturbing the genome which is important for human clinical applications, but it is also truer to the natural differentiation process where factors from the surrounding cells as well as the developing oocytes themselves and the surrounding follicular cells all affect the ultimate differentiation of the germ cells. Such key factors which can be used to induce development of follicles and IVM include Activin A and Follicle Stimulating Hormone (FSH) (9), both of which are important reproductive hormones. Activin A, a member of the transforming growth factor beta (TGF-β) family, promotes oocyte maturation in ovaria and modulates FSH activity and has been shown to enhance follicle development in vitro (10,11). FSH is a key regulator of the menstrual cycle and stimulates the development of immature follicles (12). Human Chorionic Gonadotropin (HCG) and Luteinizing Hormone (LH) are two additional factors than can be used to stimulate follicle development (4). HCG is produced in humans in the placenta during pregnancy and exercises an important function in the maintenance hereof through a feedback mechanism together with estrogen and progesterone (13). Interestingly, despite HCG only functioning after oocyte fertilization and implantation, the addition of HCG to IVM media has been noted to improve the maturation efficiency as well as the quality of the in vitro matured oocytes (14). LH is critical for ovulation in vivo (15) and thus also important for the in vitro recapitulation of follicular development and ovulation. LH too has been shown to benefit IVM of oocytes (16).

Aside from in vitro culture in the presence of specific factors, primordial germ cells or oocytes can also be co-cultured in an artificial reconstituted ovarioid (rOvaroid) (17). An rOvaroid consists of the somatic cells of an ovary reaggregated together with oocytes. This is a potentially attractive culturing system due to the high degree of fidelity that it retains to the natural developmental process that the primordial follicles undergo in the ovaries. The disadvantages of such a system are that it is unclear what factors exactly function on the developing primordial follicles, and that the somatic ovarian cells of a different individual may need to be used.

In vitro oocyte differentiation has been relatively successful in mouse studies, where fertile offspring have been born using oocytes generated from both mouse female embryonic stem cells (ESCs) and even male mouse induced pluripotent stem cells (iPSCs) (18). Promisingly, a study performed on human primordial follicles isolated from adult ovaria has succeeded in generating metaphase II oocytes that emitted polar bodies and showed a normal chromatin morphology, although the polar bodies were abnormally large (9). Although the material in this study came from adult women, the developmental stage of the oocytes that were matured in vitro is similar to the developmental stage of oocytes present in pediatric ovaries.

In pediatric ovaries, oocytes are arrested at the prophase of the first meiotic division (Figure 1) (19). At this stage, the oocytes are in a primordial follicle. During puberty, hormones such as FSH begin to affect the ovaries which leads to follicle stimulation and ovulation during menstrual cycles. The critical part of our study consists of bridging the gap between the pre-pubescent

primary oocytes that have not yet completed Meiosis I and the subsequent developmental stages where follicular development and resumption of meiosis occur.

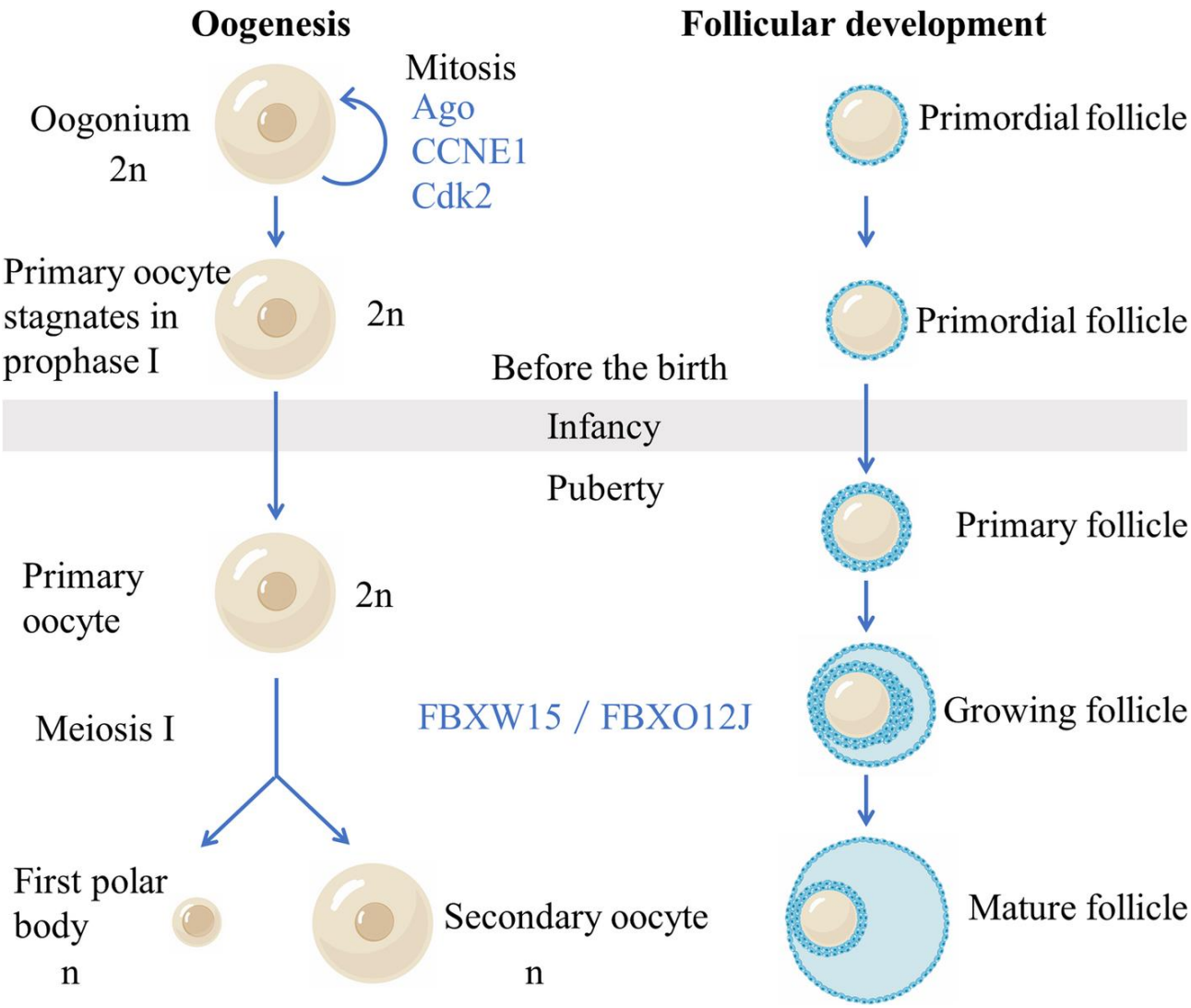

Figure 1. *Overview of the developmental stages of oocytes during oogenesis. Adapted from* (19).

It should be noted that the verification of correct functioning of oocytes is more difficult with human oocytes than it is with murine oocytes. In mouse studies, the function of the matured oocytes can simply be tested by fertilizing the oocytes and then testing whether fertile offspring are produced. Such a direct functional assay is not possible with human oocytes due to ethical concerns and legal restrictions. Instead, the investigator must rely on morphological parameters such as the correct segregation of chromosomes leading up to metaphase II, which is the final pre-fertilization stage of the oocyte, as well as the emission of correctly sized polar bodies.

Our aim is to integrate the currently available knowledge and adapt and refine the approach used to maturate human primordial follicles to enable the IVM of primordial follicles derived from cryopreserved pediatric ovaries. Using samples of cryogenically preserved ovaries obtained with permission and informed consent, we will generate metaphase II oocytes with normal morphologic features such as the correct arrangement of chromatin and the presence of the first polar body. We aim to isolate primordial follicles from the cryopreserved material and then identify the optimal mixture of growth factors and hormones that enables IVM of the cumulus-oocyte-complexes (COC), a mass of cells including the oocyte that leaves the follicle following ovulation. Preferably, we will work with serum free and xeno free reagents to facilitate the translation of our approach to the clinic.

The maturation of pediatric oocytes in vitro has not been achieved before to our knowledge. Through combining the existing knowledge and recent developments in the field of oocyte differentiation and culture, we can finally address an issue that has persisted for the past decades, allowing us to preserve fertility in female pediatric cancer patients who would otherwise remain sterile.

B.2.2 Approach

The techniques that will be employed in our study consist of tissue- and cell culture, bright field and fluorescence microscopy and in vitro oocyte maturation. These are relatively simple accessible and routinely used methods in cell biology and embryology, and they are easily applicable to achieve our aims. The more intricate part of our study will consist of the administration of specific human recombinant factors and hormones which are commercially available. Additionally, we will culture rOvaroids which will require the use of cell sorting or the removal of follicles from adult human ovaries, followed by reaggregation of the cells into clusters called rOvaroids together with oocytes from primordial follicles. We will use 3-dimensional cell culture through the use of scaffolds such as alginate to further improve the efficiency of follicle culture.

The starting point of our study is cryopreserved ovarian material from female pediatric cancer patients. A small portion of cryopreserved ovaries may be recovered for further experimentation. This material will be thawed and dissected into fragments which can be cultured further. After an 8-day period of fragment culture, follicles can then be isolated from the fragments and cultured further for 8 days before COC isolation. Once COCs are isolated, we will proceed to a systematic approach of testing various factors that stimulate oocyte development for IVM as described in previous studies (4,9,17). Following the conclusion of the experiment, oocytes will be retrieved for confocal microscopy imaging and analysis of morphology. Additionally, rOvaroid co-culture with primordial follicles will be performed. The culturing protocol includes the use of a scaffold such as alginate or oECM2, an artificial ovarian extracellular matrix (ECM) obtained from bovine ovaries (5,6).

Detailed Work Plan

Thawing of cryopreserved material & fragment culture

The cryopreserved material will be thawed for 30 seconds at room temperature, followed by rapid thawing for 2 minutes in a 37 degree Celsius water bath. Hereafter, the tissue will be washed in pre-warmed freezing solution with sequentially decreasing concentrations of the cryoprotectant (for example 1.0M, 0.5M and then 0.2M), with each step being performed for 5 minutes. The tissue will then be transferred to fresh, pre-warmed dissection medium (Leibovitz's L-15 medium supplemented with 2mM Sodium Pyruvate, 2mM Glutamine, 3 mg/mL human serum albumin (HSA), 75 microgram/mL penicillin G, 50 microgram/mL streptomycin (4,9).

The ovarian cortex which contains the immature, primordial follicles will then be dissected under a microscope into fragments approximately 1 x 1 x 0.5 mm^3 in size.

Fragments can be incubated individually in 24-wells plates in 300 microliters of culturing medium (McCoy's 5A medium with bicarbonate supplemented with 20 mM HEPES, 3 mM Glutamine, 0.1% HSA, 0.1 mg/mL penicillin G, 0.1 mg/mL streptomycin, 2.5 µg/mL transferrin, 4 ng/mL selenium, 10 ng/mL human insulin, 1 ng/mL human recombinant FSH (rhFSH) and 50 µg/mL ascorbic acid (9) for 8 days at 37 degrees Celsius with 5% CO_2, with half of the medium being replaced every 2 days. Optionally, the fragments can be cultured in a 3-dimensional scaffold such as alginate or oECM2. At the start and end of this culture period, 2+ fragments can be isolated for imaging to ascertain the number of primordial follicles present in the isolated material.

Isolation and culture of follicles and COCs

Following fragment culture, enlarged (up to 150 µm) follicles will be isolated using a needle as described by (9). Isolated follicles will be transferred into a V-bottomed 96-well plate for further culture in 150 µL of culture medium supplemented with 100 ng/mL human recombinant Activin A and 1ng/mL rhFSH. The follicles can be individually cultured for another 8 days at 37 degrees Celsius with 5% CO_2, with half of the medium being refreshed every 2 days. Hereafter, COCs will be retrieved by aspiration from intact, healthy follicles with a visible antral cavity. COCs with adherent granulosa cells will be isolated for IVM.

In vitro maturation

Varying combinations of factors have been used for IVM of human and murine oocytes (4,7,9). To refine the existing protocols for IVM, we will test different combinations and concentrations of these factors and then refine our protocol. Following COC isolation, COCs will be placed into maturation medium according to Table 1 and then cultured a further 24 – 48 hours at 37 degrees Celsius, 5% CO_2.

Table 1. *An overview of the proposed combination of media for IVM. Media available from CooperSurgical.*

Mix #	Medium	Supplement
1	SAGE IVM	75 mIU/mL FSH & 75 mIU/mL LH
2	SAGE IVM	75 mIU/mL FSH & 10 mIU/mL HCG
3	SAGE IVM	75 mIU/mL FSH & 100 mIU/mL HCG
4	SAGE IVM	75 mIU/mL FSH & 1000 mIU/mL HCG
5	Quinn's Cleavage	75 mIU/mL FSG & 10 mIU/mL HCG
6	Quinn's Cleavage	75 mIU/mL FSG & 100 mIU/mL HCG

7	Quinn's Cleavage	75 mIU/mL FSG & 1000 mIU/mL HCG

Microscopy analysis & oocyte validation

Following IVM, oocytes may be retrieved and fixed before proceeding to staining for confocal microscopy. Staining will include microtubules, microfilaments and chromatin as the correct arrangement of the metaphase-II spindle and chromatin is crucial for oocyte developmental capacity. For example, the distance between pericentriolar materials in oocytes can function as a measure of maturation competence in metaphase II oocytes (20). In any case, the oocytes will be examined for euploidy, absence of spindle abnormalities, and the emission of polar bodies. Close attention will be paid to the size of polar bodies which has been documented as being abnormally large following IVM of primordial follicles (9). This morphological examination will be supplemented by examining various biomarkers that are indicative of oocyte developmental capacity. Markers of meiosis, a vital step in the maturation of oocytes, such as SYCP1 and SYCP3 can be examined by indirect immunofluorescence for presence and correct localization (21). To further ascertain the developmental competence of the obtained oocytes, reverse transcription-polymerase chain reaction (RT-PCR) can be used to analyse the expression of biomarkers in the cumulus cells surrounding the oocyte in the COC. Generally, the level of transcription in human cumulus cells decreases as the oocyte matures. Key signalling pathways are also altered, including but not limited to cell cycle regulation, steroid metabolism, apoptosis and ECM remodelling (22). Two factors that have been associated with oocyte maturity and developmental competence are growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) (23). These factors, which are expressed in the oocyte and the cumulus granulosa cells, play an important role during oocyte development. Oocytes coming from COCs where the cumulus granulosa cells have shown relatively higher GDF9 and BMP15 expression showed better pregnancy outcome in female patients undergoing IVF, and thus the levels of their expression may also be predictive of oocyte quality in IVM derived oocytes. Because euploidy is of particular importance during IVM and because past studies have reported significant numbers of aneuploid oocytes following IVM (24), it is also worthwhile to examine biomarkers that are associated with euploidy in maturing oocytes. Pregnancy associated plasma protein A (PAPPA) is one such gene, having a modulatory function in the insulin like growth factor pathway (25). High PAPPA mRNA levels have been associated with mature oocytes and successful live births in female patients when compared with aneuploid embryos and immature oocytes.

rOvaroid culture & 3-dimensional scaffolds

In the event that the original approach reaches a low oocyte maturation percentage, we will culture and then maturate COCs derived from follicles grown in rOvaroids. rOvaroids will be created by removing the follicles from adult human ovarian material, sorting the somatic ovarian cells (SOCs) and then reconstituting them together with primordial follicles obtained from pediatric ovaries. A recent promising development is the successful generation of ovarian granulosa-like cells from human iPSCs (26). These cells, generated from human iPSCs, have been previously successfully aggregated into rOvaroids after introduction of human primordial-like germ cells (26). This would potentially enable us to produce rOvaroids without needing human ovarian material. Furthermore, it opens the possibility of using the patient's own cells to generate patient specific rOvaroids which can then be used to grow the patient's follicles in vitro. The granulosa cells of the ovaria are responsible for producing hormones such as estrogen and progesterone and may be particularly important for the in vivo development of follicles, and these should be concentrated in the rOvaroids. On the other hand, the ovarian fibroblasts may provide ECM factors which can also be important for correct follicular development (5,6), and thus their presence in the rOvaroids may support correct follicle growth and rOvaroid structure. rOvaroids combined with primordial follicles from pediatric ovaries can be cultured together in culture medium supplemented with 1 ng/mL rhFSH and 100 ng/mL Activin A. To facilitate the formation of a reconstituted ovarian organoid, the reaggregated cells can alternatively be implanted into a 3-dimensional scaffold such as alginate or a mixture of alginate and oECM2. Following follicle culture in rOvaroids, COCs can again be retrieved by aspiration from healthy antral follicles that have formed within the rOvaroids. IVM can then be attempted again to examine whether rOvaroid culture of follicles has a positive impact on the efficiency of IVM as well as the overall success rate of deriving mature oocytes from primordial follicles. Overall, the generation of (patient specific) rOvaroids integrated into an artificial ECM presents a novel, promising approach to Ovarian bioengineering and holds potential for successful in vitro follicle culture and subsequent IVM.

Duration and timeline

The duration of a single round of experimentation is estimated as follows:

Day 0: Thawing & fragment culture

↓

Day 8: Isolation and culture of follicles

↓

Day 16: Isolation of COCs, in vitro maturation start

↓

Day 16 – Day 18: In vitro maturation

↓

Day 17 – Day 18: Fixation, staining and confocal microscopy analysis, additional biomarker tests

From thawing of cryopreserved material to imaging and analysis, the protocol is expected to take 3 weeks to complete. Considering that the protocol will need to be refined and that multiple attempts may be necessary along with technical difficulties, the study is expected to last 12 – 18 months. Furthermore, for rOvaroid culture, the protocol is expected to last slightly longer to take into account the cell sorting, reaggregation and culture of rOvaroids containing foreign immature follicles. An overview of the planning for the project can be seen in Figure 2. Approximately one month will be required to plan the experiments and acquire the necessary reagents, after which the first 3 rounds of experimentation can be commenced. Each round will involve the entire process from thawing and fragment culture to IVM, imaging and analysis. After the end of each round, the efficiency of the used methods (reflected by the efficiency of IVM) will be evaluated so that gradual improvements can be made and the most optimal media for IVM can be selected. For the initial proposal, 3 blocks of 3 experiments each are planned, with evaluation of our results during the end of each block of experiments. Following the conclusion of our main approach, we will culture rOvaroids. As rOvaroid culture is presumed to take longer due to the required assembly of rOvaroids, we have planned these experiments in two blocks of two round each, lasting one month per round. After each round, we will evaluate our methods to further improve the viability of our rOvaroids and the efficiency of IVM following rOvaroid culture. Lastly, time is reserved for analysing our results as a whole and writing a paper. The planned 13 months of the project can be extended if further rounds of experimentation are necessary.

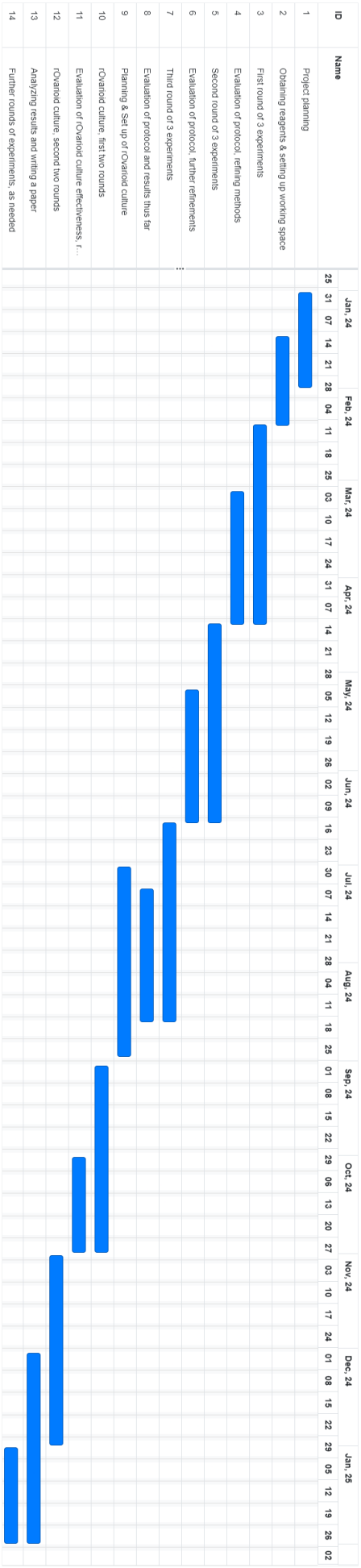
ID
Name
1 Project planning
2 Obtaining reagents & setting up working space
3 First round of 3 experiments
4 Evaluation of protocol, refining methods
5 Second round of 3 experiments
6 Evaluation of protocol, further refinements
7 Third round of 3 experiments
8 Evaluation of protocol and results thus far
9 Planning & Set up of rOvarioid culture
10 rOvarioid culture, first two rounds
11 Evaluation of rOvarioid culture effectiveness, f...
12 rOvarioid culture, second two rounds
13 Analyzing results and writing a paper
14 Further rounds of experiments, as needed
Jan, 24
Feb, 24
Mar, 24
Apr, 24
May, 24
Jun, 24
Jul, 24
Aug, 24
Sep, 24
Oct, 24
Nov, 24
Dec, 24
Jan, 25

Figure 2. *Gantt Chart of the proposed project. Created with onlinegantt.com.*

B.2.3 Feasibility / Risk assessment

The foremost challenge in our study is the limited availability of pediatric ovarian material. Because the source material is cryopreserved for former cancer patients in order to potentially preserve their fertility, we will only be able to work with a part of each cryopreserved ovary, 15 % to be precise, which necessitates careful utilization of our resources. A positive aspect of our approach is that for follicle culture, we will fragment out samples into numerous small fragments which, following culturing, will be split up into even more isolated follicles. If the number of primordial follicles available per fragment is sufficiently high, we will also be able to work with less material per experiment to preserve the amount of ovarian tissue available.

This is particularly essential as we will need to refine our protocol and determine which combination of maturation medium and hormone supplements is the most efficient to achieve conversion of immature oocytes to morphologically normal and developmentally competent metaphase-II oocytes.

Another issue is presented by the origin of our samples as cryopreserved material from pediatric cancer patients. Cryopreserved material from cancer patients can carry malignant cells (27). For this reason, any ovaries that are to be reimplanted into women that have undergone cancer treatment must be thoroughly screened for malignant cell infiltration, malignant cell residue (MRD). We will isolate only the COCs, focusing on the maturation of the oocyte. Thus, the end product of a successful experiment, a metaphase-II oocyte, could be employed in IVF without carrying the risk of reintroducing malignant cells into the patient. This issue is similarly bypassed in the rOvaroid culture approach as only the primordial follicle is retrieved from the original cryopreserved material, and eventually only the COC is isolated as well. Our approach thus removes the risk carried by MRD.

Perhaps the most challenging aspect of our study is the full recapitulation of follicular development and oocyte maturation in vitro. This procedure is successful in mice (17), but has only been reported once with human follicles before (9). Furthermore, the human study in question used adult ovaries rather than pediatric ovaries. Still, our approach has merit due to working with follicles that are in a similar stage of development as the follicles used in the human study. Furthermore, IVM of human oocytes from advanced developmental stages is more well documented and various media and hormone supplements which support IVM have been identified (4). This leads us to conclude that it is possible to induce IVM of oocytes derived from pediatric ovaries for the first time.

Alternatively, we will generate rOvaroids from somatic cells of adult human ovaries. Reaggregating these cells together with the primordial follicles from pediatric ovaries will enable us to simulate the in vivo conditions that arise in the ovaria during and after puberty, thereby stimulating the natural development of primordial follicles. If adult ovaries are unavailable, we can also generate rOvaroids from iPSC derived ovarian granulosa-like cells.

B.2.4 Scientific (a) and societal (b) impact

The short-term benefits of our research include a further elucidation of the factors required for IVM of human oocytes. This will enable researchers who work with human follicles and oocytes to achieve higher efficiency of IVM, preserving time and the often expensive, short shelf-life media which are required for IVM of oocytes. Importantly, a higher efficiency of follicle development and IVM will also allow less of precious cryopreserved ovarian material to be used in other studies. Lastly, characterization of factors necessary for full in vitro development of pediatric primordial follicles to metaphase II oocytes will make this research area more approachable within the field, strengthening our understanding of the requirements for the development of female human gametes.

In the long term, achieving full in vitro development of oocytes will more clearly illustrate the differences in requirements between human and mouse oocytes, which may in turn lead to more successful translation of studies performed in mice to studies performed with human follicles and oocytes. The prospect of generating developmentally competent metaphase-II oocytes from, for example, human iPSCs derived from skin fibroblasts, is quite exciting. Not only would this be a major step in fertility preservation research, but it would also enable more studies focussing on female gamete development as it would be possible to generate mature oocytes from iPSCs rather than having to obtain them from sometimes scarcely available cryopreserved material. Enabling the in vitro development of human primordial follicles and then the IVM of oocytes from these follicles is a key step that needs to be successfully completed before it is possible to generate human developmentally competent metaphase-II oocytes in vitro from human iPSCs. The successful culture of a patient derived rOvaroid integrated into artificial ovarian ECM and holding patient derived primordial follicles would be a major success for tissue engineering and regenerative medicine by setting another step towards bioengineered ovaries.

The societal impact of our study would be broad for multiple groups of patients. Firstly, achieving the in vitro development of metaphase-II oocytes from primordial follicles derived from pediatric ovaries would for the first time offer the possibility of IVF for women who have endured pediatric cancer and had to have their ovaries excised due to undergoing a potentially

sterilizing cancer treatment. If our study is successful, we would have generated a protocol specifically tailored to this end which would enable clinical follow up studies investigating the possibility of using our protocol to produce metaphase-II oocytes which would then be used for IVF. Thus, the unmet medical need of fertility preservation for female pediatric cancer patients would have finally been met.

The benefits of our research will not be isolated to female cancer patients, however. As mentioned above, successfully generating metaphase-II oocytes from human primordial follicles is a key hurdle that must be overcome if we are to generate female gametes from human iPSCs. In turn, this would enable women who are infertile for any different reason to undergo a fertility restoration procedure by generating mature oocytes from, for example, skin fibroblasts. Lastly, an interesting concept made possible by our line of research is the generation of female gametes from primordial follicles derived from male iPSCs. This concept which was already successfully performed in mice (17) could allow bipaternal fertilization in the future.

B.2.5 Ethical considerations

Our study makes use of frozen human material, and thus it is important to discuss ethical considerations. Experimentation with pediatric material entails unique ethical problems because consent may at first be given by the parents of the patient when the patient is young, but when the patient reaches adulthood, this may change. The communication about fertility preservation treatments and the use of cryopreserved material for scientific research can be difficult for this reason as fertility preservation is an ongoing effort that continues even after the pediatric cancer patients undergo treatment (28). Relevant permission and informed consent of the patient/parents of the patient needs to be available. Because the samples that we will use were meant for fertility preservation, we will also only be able to use a limited amount (in essence 15%) instead of the entire ovary for each sample.

A different ethical problem is presented by the end product of our study. If we succeed, we will have generated mature metaphase-II oocytes which are theoretically prepared for fertilization. Fertilizing oocytes generated in this way is a common method to assess their function and the efficiency of IVM in mouse studies, but this is legally not possible for human oocytes. Thus, in order to nonetheless assess the efficiency of IVM without the possibility of fertilizing the generated oocytes, we will have to resort to morphological assays as well as verifying the characteristics of fertile oocytes such as the emission of polar bodies and euploidy. The generation and usage of potentially fertile oocytes in this way may in itself be viewed as an ethically sensitive procedure. The success of our study would yield additional ethical questions. If our study facilitates the generation of functional human oocytes from iPSCs, then further ethical problems will arise from the generation and usage thereof. Lastly, the combination of somatic ovarian cells of an adult woman with the primordial follicles of a different female in rOvaroids needs ethical discussion. Not only is it important to thoroughly remove any follicles from the original ovaries and obtain consent for the use of these adult ovaries in this way, but if such a protocol was followed to allow fertility preservation for pediatric cancer patients, it is essential to source adult human ovaries on a larger scale which is challenging. This challenge could be overcome by employing patient iPSC-derived ovarian granulosa-like cells to generate rOvaroids.

One additional issue is the legal status of IVM in the Netherlands. Currently, procedures involving IVM, that is the maturation of reproductive cells outside of the body, are only permitted during scientific research (29). Until recently, this was viewed in the context of the current advancement of this research field because little success was had with the complete generation of human gametes in vitro. However, with the advent of our study, IVM as a clinical procedure for the fertility preservation of former pediatric cancer patients will become a tangible possibility. Therefore, in order to utilize the benefits that our study may provide, the laws concerning this area of fertility preservation practices will need to be reviewed and updated.

B.2.6 Literature/references

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