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THE HUMAN OVARY

A CHARACTERIZATION OF CELL TYPES AND ADVERSE OVARIAN SIDE EFFECTS OF CHEMOTHERAPY

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THE HUMAN OVARY:

A CHARACTERIZATION OF CELL TYPES AND ADVERSE OVARIAN SIDE EFFECTS OF CHEMOTHERAPY

Thesis for Doctoral Degree (Ph.D.)

By

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This thesis is dedicated to all the patients who have selflessly donated tissues to support research and science, aware it is future generations that will benefit.

Also, this thesis is dedicated wholeheartedly to my family for their endless support and encouragement. My parents who instilled in me my curiosity and longing for sincerity. My sisters whose love I draw my strength from. My grandparents who inspired me to pursue a PhD.

ABSTRACT

The human ovary has a major role in the body's endocrine and reproductive system. Understanding its cell type composition is crucial in order to study underlying mechanisms of hormonal balance and ovarian follicle maturation in health and disease. The outer lining of the ovary, the cortex, harbors the ovarian reserve. This reserve is made up of follicles that have formed before birth. The prevailing dogma of female fertility sees this reserve as being limited in humans, with follicle numbers decreasing over time. Once the ovarian reserve is depleted, women enter menopause which marks the end of their reproductive period. Premature ovarian insufficiency is characterized by an early depletion of the ovarian reserve and reveals itself, among others, with an altered hormone profile and early menopause onset. Anti-cancer treatment is one of the causes of premature ovarian insufficiency. However, mechanisms underlying chemotherapy-induced altered ovarian tissue morphology and function are not yet fully understood. Therefore, effective treatments to prevent or cure the damage are still missing. One possibility for novel treatments could be offered by oogonial stem cells (OSCs) that have been recently reported to exist in the adult human ovaries. These cells can be isolated with DDX4 antibody (Ab), show a gene expression profile similar to germline stem cells in the developing embryo and have the potential to mature into oocytes.

The overall aim of this thesis is to assess the effect of first-line chemotherapy on ovarian follicles and stroma by using a tissue collection from fertility preservation programs and to dissect the structure of human ovarian cortex at single-cell resolution by using advanced single-cell profiling technology.

In study I, the effects of first-line chemotherapy that is not considered a risk for future fertility on ovarian follicles and stroma cells from pediatric and young adult ovaries were investigated. Exposure to chemotherapy prior to ovarian tissue cryopreservation revealed an impact on follicle and stroma health. Early ovarian follicles (primordial and intermediary) appeared more atretic and were smaller, while healthy growing follicles (primary) decreased in numbers. Steroid production by treated ovarian tissue in culture decreased and DNA damage as well as fibrotic lesions in the ovarian stroma increased.

In study II, we assessed the cellular composition of the adult human ovarian cortex at a single-cell level. Transcriptome and surface proteome analysis revealed six major cell types consistently detected in the ovarian cortex from Cesarean section and gender reassignment patients, namely oocytes, immune cells, granulosa cells, endothelial cells, perivascular cells and stroma cells. In order to investigate the existence of the previously reported OSCs, ovarian cortex cells positive for DDX4 Ab were isolated and analyzed. DDX4 Ab-positive cells did not show a germline-like profile, neither at a transcriptional nor protein level, but instead could be identified as perivascular cells. Furthermore, when integrating published single-cell sequencing

data from fetal germ cells, we could not identify any cortex cell with a transcriptome profile of germline stem cells.

In conclusion, we demonstrate that first-line chemotherapy treatment has negative effects on the health of ovarian cortex and exposure should be limited before cryopreservation of ovarian tissue, if possible. Adult ovarian cortex is made up of various cell types, but no evidence for the existence of germline-like stem cells was found.

WISSENSCHAFTLICHE ZUSAMMENFASSUNG AUF DEUTSCH

Der menschliche Eierstock spielt eine wichtige Rolle im Hormonhaushalt und in der Fortpflanzung. Um die zuständigen Mechanismen der Hormonproduktion und der Reifung der Eizellen in Bezug auf Gesundheit und Krankheit untersuchen zu können, ist es entscheidend, die zugrunde liegende Zell-Zusammensetzung des Eierstocks zu verstehen. Der äußere Teil des Eierstocks, der Kortex, beherbergt die sogenannte "Eierstockreserve", die aus Follikeln besteht (ein Follikel enthält eine Eizelle, die umgeben ist von Helferzellen, auch Granulosazellen genannt), die bereits vor der Geburt im Embryo angelegt wurden. Das vorherrschende Dogma der weiblichen Fruchtbarkeit sieht diese Reserve als begrenzt an, wobei die Follikelzahl mit der Zeit drastisch abnimmt. Sobald die Eierstockreserve erschöpft ist, treten Frauen in die Wechseljahre ein, was das Ende ihrer Fortpflanzungsperiode bedeutet. Die Krebsbehandlung ist eine der Ursachen für eine vorzeitige sogenannte Ovarialinsuffizienz, für die eine drastische Verringerung der Eierstockreserve charakteristisch ist und die sich unter anderem mit einer veränderten Hormonproduktion und dem sehr frühen Beginn der Wechseljahre bemerkbar macht. Die Mechanismen, die der durch Chemotherapie induzierten Veränderungen im Eierstock zugrunde liegen, sind jedoch noch nicht vollständig verstanden. Neuere Studien berichten über die Existenz von sogenannten oogonialen Stammzellen (OSCs), die mithilfe von DDX4-Antikörpern isoliert werden können. Diesen Studien zufolge ähneln OSCs den Keimbahnstammzellen im sich entwickelnden Embryo und haben das Potenzial, zu Eizellen zu reifen. OSCs wurden als mögliche Behandlungsmethode in der künstlichen Befruchtung in Betracht gezogen, und bereits an bestimmten Kliniken angeboten.

Das übergeordnete Ziel dieser Arbeit war es, die Wirkung der Chemotherapie auf Eizelle und Stroma herauszuarbeiten, sowie den menschlichen Eierstock in seiner Zell-Zusammensetzung zu verstehen.

In Studie I wurden die Auswirkungen einer Chemotherapie auf Follikel und Stromazellen von Eierstöcken bei Kindern und jungen Erwachsenen untersucht. Die Behandlung mit Chemotherapie vor der Kryokonservierung des Eierstockgewebes zeigte einen negativen Einfluss auf die Gesundheit der Follikel und des Stromas. Unreife Follikel schienen atretischer und kleiner zu sein, während die Anzahl der gesunden, wachsenden Follikel abnahm. Die Hormonproduktion von Eierstockgewebe in Kultur nahm ab und DNA-Schäden sowie fibrotische Läsionen im Stroma nahmen zu.
In Studie II haben wir die zelluläre Zusammensetzung des adulten menschlichen Eierstock-Kortex` auf Einzelzellebene untersucht. Die Transkriptom- und Oberflächenproteomanalyse ergab sechs Hauptzelltypen, die im Eierstock-Kortex von Patienten mit Kaiserschnitt und Geschlechtsumwandlung konsistent nachgewiesen werden konnten. Die sechs identifizierten Zelltypen sind Eizellen, Immunzellen, Granulosazellen, Endothelzellen, perivaskuläre Zellen

und Stromazellen. Um die Existenz der zuvor berichteten OSCs zu untersuchen, wurden DDX4 Antikörper-positive Zellen isoliert und analysiert. DDX4 Antikörper-positive Zellen zeigten weder auf Transkriptions- noch auf Proteinebene ein Profil, welches Keimbahnzellen ähneln würde. Allerdings konnten sie als perivaskuläre Zellen identifiziert werden. Perivaskuläre Zellen sind verantwortlich für die Stabilisation und Kontraktion von Blutgefäßen. Darüber hinaus – und unabhängig von DDX4 Antikörper-positiven Zellen – konnten wir keine einzelne Zelle im adulten Kortex identifizieren, die den Keimbahnstammzellen eines Embryos ähneln würden.

Zusammenfassend zeigen wir, dass eine Chemotherapie negative Auswirkungen auf die Gesundheit des Eierstockgewebes hat und soweit möglich begrenzt werden sollte bevor die Patientin Teile des Eierstocks kryokonserviert. Der adulte Eierstock besteht aus sämtlichen verschiedenen Zelltypen, allerdings wurden keine Hinweise auf die Existenz keimbahnähnlicher Stammzellen gefunden. Somit konnte hier das vorherrschende Dogma einer begrenzten Eierstockreserve bestätigt werden.

SCIENTIFIC PAPERS INCLUDED IN THE THESIS

I. Pampanini V.[*], **Wagner M.**[*], Asadi-Azarbaijani B., Oskam I., Sheikhi M., Sjödin M., Lindberg J., Hovatta O., Sahlin L., Duque Björvang R., Otala M., Damdimopoulou P.[**], and Jahnukainen K[**].
Impact of first-line cancer treatment on the follicle quality in cryopreserved ovarian samples from girls and young women.
Human Reproduction 2019 Sep 29;34(9):1674-1685.

Appendix: Reply: Impact of first-line cancer treatment on follicle quality in cryopreserved ovarian samples. Human Reproduction 2020 Apr 13; 35(5): 1249-1251.

II. **Wagner M.**, Yoshihara M., Douagi I., Damdimopoulos A., Panula S., Petropoulos S., Lu H., Pettersson K., Palm K., Katayama S., Hovatta O., Kere J., Lanner F.[**], Damdimopoulou P.[**]
Single-cell analysis of human ovarian cortex identifies distinct cell populations but no oogonial stem cells.
Nature communications 2020 Mar 02;11(1):1147.

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ADDITIONAL PUBLICATION NOT INCLUDED IN THE THESIS

I. Johansson H., Damdimopoulou P., van Duursen M., Boberg J., Franssen D., de Cock M., Jääger K., **Wagner M.**, Velthut-Meikas A., Xie Y.,m Connoly L., Lelandais P., Mazaud-Guittot S., Salumets A., Kam Draskau M., Filis P., Fowler P., Christiansen S., Parent AS., Svingen T.
Putative adverse outcome pathways for female reproductive disorders to improve testing and regulation of chemicals.
Archives of Toxicology 2020, *accepted*

CONTENTS

LIST OF ABBREVIATIONS

AMH	Anti-Müllerian hormone
ASCO	American society of clinical oncology
AUGMENT	Autologous germline mitochondrial energy transfer
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BrdU	5-Bromodeoxyuridine
C-KIT	v-kit Hardy Zuckerman 4 feline sarcoma viral oncogene homolog Anti-Müllerian hormone
C-sec	Cesarean section
CED	Cyclophosphamide equivalent dose
CFU	Colony forming unit
CYP11	Cytochrome P450, family 11, subfamily A, polypeptide1, also known as Desmolase
CYP17	Cytochrome P450, family 17, subfamily A, polypeptide1
CYP19	Cytochrome P450, family 19, subfamily A, polypeptide1, also known as Aromatase
CYP26B1	Cytochrome P450, family 26, subfamily B, polypeptide1
DAPI	4’,6’-Diamidino-2-phenylindole
DAZL	Deleted in azoospermia like
DDX4	DEAD (Asp-Glu-Ala-Asp)-box polypeptide 4; also known as VASA
DIE	Isotoxic dose equivalent
DPPA3	Developmental pluripotency associated 3
DSB	Double strand break
EGF	Epidermal growth factor
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FGC	Fetal germ cell
fMSC	Fetal mesenchymal stem cells
FOXL2	Forkhead box L2
FOXO3A	Forkhead box O3
FSH	Follicle stimulating hormone

FSHR	Follicle stimulating hormone receptor
GDF	Growth differentiation factor
γH2AX	H2A histone family member X phosphorylated in serine 139
GnRH	Gonadotropin releasing hormones
GRP	Gender reassignment patient
HEK	Human embryonic kidney
HPG	Hypothalamic-pituitary-gonadal
HSA	Human serum albumin
ICSI	Intracytoplasmic sperm injection
IFITM3	Interferon induced transmembrane protein 3
IHC	Immunohistochemistry
IPSC	Induced pluripotent stem cell
IVF	*In vitro* fertilization
KITL	KIT ligand, also known as Stem Cell Factor
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LIF	Leukemia inhibitory factor
MACS	Magnetic-activated cell sorting
MS	Mass-spectrometry
MTORC1	Mammalian target of rapamycin complex 1
MSC	Mesenchymal stromal cell
MVH	Mouse vasa homolog
OSC	Oogonial stem cell
OTT	Ovarian tissue transplantation
PFA	Paraformaldehyde
PGC	Primordial germ cell
PI3K	Phosphoinositide 3-kinase
POU5F1	POU class 5 homeobox1; also known as OCT3/4
PRDM1	PR domain zinc finger protein 1
PRDM14	PR domain zinc finger protein 14
PTEN	Phosphatase and tensin homolog
PUMA	P53 upregulated modulator of apoptosis
QRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RA	Retinoic acid
RpS6	Ribosomal protein S6

S6K	Ribosomal protein S6 kinase
ScRNA-seq	Single-cell mRNA sequencing
SOX2	SRY-box transcription factor 2
SSEA-4	Stage specific embryonic antigen 4
STRA8	Stimulated by retinoic acid gene 8
SYCP3	Synaptonemal complex protein 3
Tap63	Isoform of p53 homolog p63
TGF-b	Transforming growth factor-beta
TSC1/2	Tuberin/tuberous sclerosis complex 1 and 2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TZP	Transzonal projections
VSELC	Very small embryonic-like cell
ZP3	Zona pellucida 3

1 INTRODUCTION

1.1 THE HUMAN OVARY

Structure and function

Ovaries are central reproductive organs in women with two major functions: production of hormones (steroidogenesis) and generation of developmentally competent mature oocytes (folliculogenesis). There are two ovaries, located on either side of the abdomen, connected to the uterus *via* the fallopian tubes (Fig.1A). The adult ovary consists of an outer part, the cortex, and an inner part, the medulla (Fig.1A, B). They are lined with an epithelial cell layer (also called human ovarian surface epithelium). The ovaries are highly plastic structures that allow for rupture and subsequent healing of the ovary surface when oocytes are being ovulated every month (Auersperg et al., 2001).

Human ovarian follicles are formed during fetal life and can remain in a dormant state of so-called primordial follicles for decades. Primordial follicles can be found in the cortical tissue and are composed of an oocyte and surrounding granulosa cells (Fig.1C). During the process of folliculogenesis (as described in more detail later), the primordial follicle becomes activated and starts to grow: the oocyte grows in size, granulosa cells proliferate, and theca cells differentiate to surround the growing follicle. During this process, the follicle migrates inwards to the medullary region of the ovary. The mature follicle finally releases the oocyte which can be fertilized by one spermatozoon to become an embryo.

During follicle growth and maturation, the oocytes are in bi-directional communication with somatic cells within the ovary, which is necessary for oocyte development and normal functioning of the ovary (Kristensen et al., 2014). Granulosa cells are in physical contact with the oocyte. Communication happens mainly *via* paracrine factors through transzonal projections (TZPs) of granulosa cells reaching the oocyte surface (El-Hayek et al., 2018). First discovered over 50 years ago (Hadek, 1965), it has now been shown that TZPs connect granulosa cells and the oocyte through adherens junctions. Adherens junctions are found in tubulin-rich TZPs. While actin-rich TZPs make up the majority of TZPs and contain gap junctions that are responsible for signaling to and nutritional support of the oocyte (Li et al., 2013). In addition, bi-directional communication is ensured *via* signaling molecules of the transforming growth factor-ß (TGF-ß) superfamily. The TGF-ß superfamily includes bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), inhibins, activins, follistatin and anti-Müllerian hormone (AMH) (Chang et al., 2016). Furthermore, the growth factor Kit ligand (KITL) is expressed by granulosa cells and signals to the oocyte *via* its receptor C-Kit, playing a key role in follicle development (Carlsson et al., 2006).

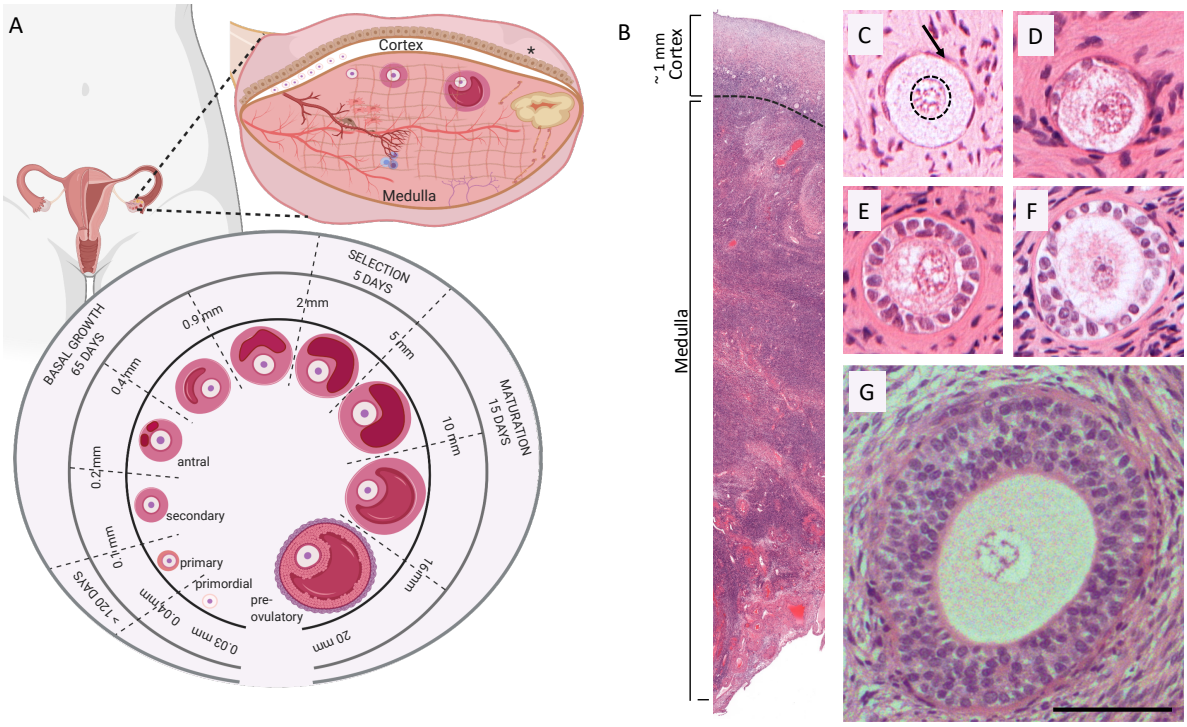

Fig.1 Physiology and anatomy of the human ovary. (A) The ovaries are connected to the uterus *via* fallopian tubes and are composed of an outer cortex and an inner medulla. A single layer of epithelial cells covers the cortical surface (marked with *). In cortex and medulla, many different cell types and structures can be found. Within the ovaries, the maturation of follicles occurs in a process termed folliculogenesis. It takes several menstrual cycles for the follicle to fully mature and reach the pre-ovulatory stage. Graphics depicting folliculogenesis adapted from (Gougeon, 1996) with permission and (A) was created using ©BioRender. (B) The ovarian cortex is approximately 1 mm thick and harbors the ovarian reserve. This reserve contains the primordial follicles arrested in a dormant state. Only upon activation, primordial follicles start to grow and mature, moving towards the more vascularized medullary region of the ovary. (C-G) The early stages of follicle maturation are shown. Ovarian tissue sections were stained with hematoxylin and eosin. (C) Primordial follicle; dotted circle surrounds the oocyte nucleus for better visualization, black arrow points at one flattened granulosa cell surrounding the oocyte. (D) Intermediary follicle with both, flattened and cuboidal granulosa cells. (E) Primary follicle with one entire layer of cuboidal cells. (F) and (G) show secondary follicles as granulosa cells proliferate and form several layers. Scale bar = 50 µm.

Besides signaling *via* growth factors, ovarian follicles are regulated by and produce steroid hormones. These hormones provide a feedback mechanism for regulation of reproduction known as the hypothalamic-pituitary-gonadal (HPG) axis (Rabinovici, 1993) (Fig.2). The HPG axis becomes activated during puberty: the hypothalamus produces gonadotropin-releasing hormones (GnRH) that act on the anterior pituitary. Here, GnRH stimulate endocrine cells to release the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Both hormones are needed to activate steroidogenesis in ovarian somatic cells, with granulosa cells and theca cells being significantly involved in steroid hormone production. LH binds to theca cells in the ovary *via* LH receptor (LHR). Theca cells further convert cholesterol into androgens, such as androstenedione and testosterone, by the enzymes desmolase (or CYP11) and CYP17. When FSH binds to granulosa cells *via* FSHR, aromatase (or CYP19) is activated and can now convert androgens into estrogens, including estradiol and estrone (Andersen et al., 2014). Upon ovulation of the oocyte, a corpus luteum is formed from the follicle remnants

(theca and granulosa cells) that secretes progesterone which is essential in preparing the uterus´ receptivity for potential embryo implantation. *Via* negative feedback, estrogen and progesterone inhibit the further production of GnRH in the hypothalamus and gonadotropins in the pituitary. When the oocyte remains unfertilized, the corpus luteum degenerates and ceasing levels of progesterone and inhibins secreted by the degenerating corpus luteum allow for a new cohort of follicles to grow and mature by the secretion of FSH from the pituitary (Gilbert, 2000).

Folliculogenesis requires several months (Williams et al., 2000) and out of million primordial follicles formed before birth, approximately 400 oocytes are being ovulated before a woman enters menopause (Wallace et al., 2010).

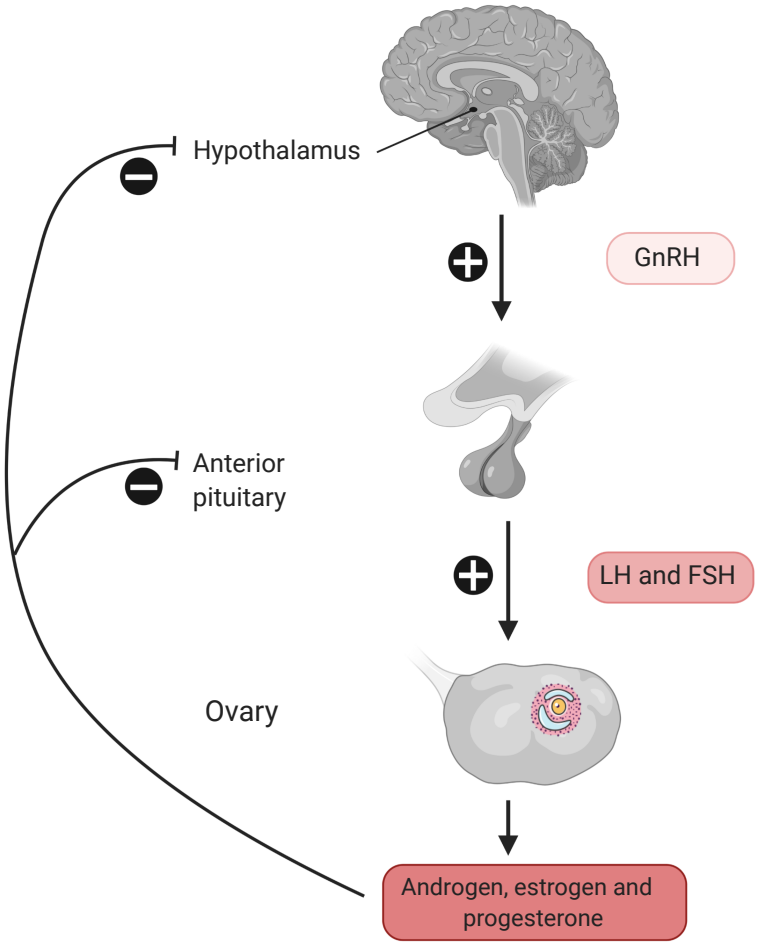

Fig.2. Hypothalamic-pituitary-gonadal axis and regulation of ovarian folliculogenesis. The HPG axis controls the menstrual cycle by regulating the release of ovarian hormones. GnRH produced in the hypothalamus stimulates the release of LH and FSH in the anterior pituitary. LH and FSH, in turn, trigger the production of androgen, estrogen and progesterone in the ovary. Progesterone and estrogen exert an inhibitory effect on hypothalamus and pituitary to downregulate further GnRH release. Graphics was created using ©BioRender.

Cell types in the human ovary

In addition to the four cell types mentioned in the previous chapter (ovarian surface epithelium cells, oocytes, granulosa cells and theca cells) various somatic cell populations are expected to be found in human ovarian stroma. For example, endothelial cells fulfill important functions in the tissue as they contribute to blood vessel formation, which is crucial for follicle growth and atresia as well as for the formation of the corpus luteum after ovulation (Christenson et al., 1996; Davis et al., 2003; Reeves, 1971; Suzuki et al., 1998b). In addition, perivascular cells,

including pericytes and smooth muscle cells, are needed where blood vessels are formed to stabilize endothelial cell structures and regulate blood flow (Wanjare et al., 2013). Lymphatic vessels are described to be part of the ovary's vascular system, closely associated with the blood vasculature. These vessels are made up of lymphatic endothelial cells and smooth muscle cells and play a role in follicle development and wound healing (Brown et al., 2014). Furthermore, the ovary is innervated by extrinsic and intrinsic neurons which are hypothesized to have roles in ovary development, regulation of blood flow and follicle maturation (Anesetti et al., 2001). Mesenchymal stromal cells (MSCs) and immune cells are reported to support follicle growth and degeneration of the follicle after the oocyte has been ovulated, as well as facilitating closure and healing of the ruptured epithelial layer after ovulation (Bukulmez et al., 2000; Ding et al., 2016a; Suzuki et al., 1998a). A recent single-cell report was the first to show the complexity of the different cell types found within the human medulla, including a variety of adaptive immune cells, such as T-cells, NK-cells, and B-cells, as well as innate immune cells, such as monocytes (Fan et al., 2019). Since the early 2000s, the existence of stem cell populations that have the potential to give rise to oocytes in the adult ovary is under continuous discussion. There are populations reported to have their origin in the cortex (putative oogonial stem cells) or in the surface epithelium of the ovary (very small embryonic-like cells, VSELCs). Their existence is still debated and will be elaborated in a later section.

1.1.1 Human germ cell development

Male and female germ cells, spermatozoa and oocytes, respectively, can fuse in the process of fertilization and give rise to a new human being. With the union of one oocyte and one spermatozoon, a zygote is formed which subsequently develops into a blastocyst that implants to the receptive uterine endometrium for further development. In the developing mouse embryo, the first primordial germ cells (PGCs, also referred to as fetal germ cells, or FGCs) can be identified shortly after implantation when they are being specified from a subpopulation of epiblast cells (Lawson et al., 1994). In humans, several hypotheses co-exist currently. Human PGCs may arise from the yolk sac endoderm, the extra-embryonic mesoderm or, like in mice and currently the most prominent hypothesis, from the posterior epiblast (Chen et al., 2017) and were detected as early as week 4-5 of gestation (Tyser et al., 2020). Upon PGC specification in the human fetus, these cells reside within the primitive streak. They start proliferating and migrate to the gonadal ridge by week 6 of human gestation. At the gonadal ridge, PGCs continue to proliferate by mitosis, characteristically expressing among others PRDM14, POU5F1, NANOG and LIN28A (Li et al., 2017), before colonizing the fetal ovaries and differentiating into oogonia (Sarraj et al., 2012). The sexual fate of PGCs is not determined by its own constitution of sex chromosomes (XX or XY) but the constitution of the fetal gonad (Adams et al., 2002), hence migrating PGCs are not determined yet in their sexual identity but only upon arrival at the fetal ovary or testis. In the fetal ovary, PGCs are now termed oogonia. Here, they continue proliferating and form clusters of interconnected cells that become

enclosed by somatic cells in a structure called germ cell nests. During this differentiation process, oogonia become retinoic acid (RA) signaling-responsive, characterized by the expression of stimulated by retinoic acid gene 8 (STRA8), before entering meiosis (Li et al., 2017). The ability to respond to RA signaling and hence initiate meiosis discriminates oogonia from spermatogonia. In fetal testes, the increased expression of CYP26B1 was reported to play a major role in quick degradation of RA to not yet initiate meiosis in spermatogonia (Bowles et al., 2006; Endo et al., 2019). By gestational week 9, oogonia express distinct germ cell markers, such as C-KIT, PRDM1, DDX4, DAZL, and SYCP3 (Eguizabal et al., 2016; Gomes Fernandes et al., 2018; Kerr et al., 2008). SYCP3, or synaptonemal complex protein 3, is a meiosis specific protein that enables oogonia to undergo meiosis. When meiosis is initiated around eleven to twelve weeks of gestation, oogonia are now termed oocytes, and the expression of pluripotency genes (e.g. POU5F1 and NANOG) is down-regulated (Anderson et al., 2007; Guo et al., 2015). By week 17, the majority of oogonia have entered meiosis. However, it has been reported that germ cells of different developmental stages (e.g. in mitotic, RA signaling-responsive, meiotic and oogenesis phase) co-exist in later stages of human embryo development. In an 18 weeks old fetus for example, all four stages of germ cells were found to be present (Guo et al., 2015; Li et al., 2017). Finally, oocytes become arrested in prophase I of meiosis I in dictyate stage when recombination of homologous chromosomes has occurred and chromosomes are still attached at chiasmata (Motta et al., 1997). Lastly, germ cell nests containing several oocytes are infiltrated by the enclosing ovarian somatic cells around week 18, resulting in nest breakdown and the formation of the first primordial follicles as they appear in the post-natal ovary: a primordial follicle made up of one oocyte arrested in meiosis I, surrounded by flattened somatic epithelial granulosa cells (Tingen et al., 2009). Oocytes can remain in this stage for decades until they are activated, resume meiosis prior to ovulation and complete meiosis upon fertilization (Cohen et al., 2010; MacLennan et al., 2015).

1.1.2 Folliculogenesis and the current dogma of limited female fertility

In 1870, the anatomist and embryologist Waldeyer initially formulated the dogma of limited female fertility in humans due to a fixed ovarian follicle reserve established before birth (Waldeyer, 1870). Since then, researchers have been and still are investigating this phenomenon that is in stark contrast to the human male fertility with 200-300 million new spermatozoa being produced on a daily basis until death (Padubidri, 2011). Women exhaust their ovarian reserve on the average by 50-51 years of age and enter menopause (Faddy et al., 1996; Faddy et al., 1992; Wallace et al., 2010), after which the chances of becoming a biological mother are lost. The dogma of a fixed follicle reserve is still considered valid: the human ovarian reserve is made up of millions of primordial follicles, established during embryo development and set before birth (Fakih, 2015). Already prenatally, a major fraction of those primordial follicles will start undergoing cell death, called follicle atresia, due to yet unknown reasons. Primordial follicle atresia is considered the main cause of constant

diminishing of the ovarian reserve throughout a woman´s life. In contrast, ovulation of mature oocytes consumes only a comparably small number of primordial follicles (Wallace et al., 2010). Continuously throughout postnatal life, dormant primordial follicles are activated and recruited into the pool of growing follicles (McGee et al., 2000). Upon puberty, the HPG axis becomes active and gonadotropins are secreted allowing a fraction of the recruited follicles to further mature. During folliculogenesis, the follicle grows in size from around 30 µm to 20 mm (Fig.1A) with the oocyte reaching 120 µm at the time of ovulation (van den Hurk et al., 2005).

Histologically, developmental stages of follicles have been well characterized (Gougeon, 1996) (Fig.1A). When primordial follicles (Fig.1C) are activated, flattened somatic pre-granulosa cells surrounding the immature oocyte become cuboidal while transitioning through the stage of an intermediary follicle (Fig.1D.). When surrounded by a full layer of cuboidal cells, the follicle is called a primary follicle (Fig.1E). These cuboidal granulosa cells proliferate to form a multi-layered secondary follicle (Fig.1F, G). An antrum is formed in the follicle that expands. Theca cells differentiate and align around the follicle to further support follicle maturation until the oocyte reaches its final state of maturation in the antral follicle. This process is called folliculogenesis, the maturation of the primordial follicle to the ovulated oocyte.

Although some pathways were discovered to play crucial roles in primordial follicle activation, the exact molecular mechanisms and interplays of involved genes are still elusive. The PI3K/AKT signaling pathway (Fig.3) was found to be involved by several independent research groups using genetically modified mouse models (Adhikari et al., 2009b; Reddy et al., 2008; Reddy et al., 2010; Zhang et al., 2014) and suspected to be equally important in bovine (Andrade et al., 2017) and human (Grosbois et al., 2018; Kawamura et al., 2013). It is to date the best studied mechanism in the mammalian ovary. Briefly, the phosphoinositide 3-kinase (PI3K) is negatively regulated by PTEN when the follicle is arrested in its dormant state. Upon binding of different ligands (e.g. KITL, Insulin) to their respective receptors (e.g. C-KIT) on the oocyte, the PI3K pathway is activated and elicits a cascade of phosphorylation of various proteins, among them AKT which phosphorylates TSC1/2 (tuberin/tuberous sclerosis complex 1 and 2). Phosphorylated TSC1/2 is inactive and cannot inhibit Rheb protein any longer which, subsequently, leads to mTORC1 activation. Activated mTORC1, or mammalian target of rapamycin complex 1, can phosphorylate S6K, a ribosomal protein kinase, which in turn phosphorylates the ribosomal protein rpS6. RpS6 initiates the synthesis of proteins involved in cellular survival and growth. It was shown in mice that the deletion of TSC1/2 in oocytes of primordial follicles leads to a premature depletion of the ovarian reserve (Adhikari et al., 2009a; Adhikari et al., 2010). Another target of AKT is FOXO3A. FOXO3A is usually residing in the cell nucleus acting as a transcriptional activator, assuring oocyte dormancy. Upon phosphorylation, FOXO3A translocates to the cytoplasm and consequently, cannot function as a transcription factor anymore. Studies using transgenic mouse models reveal a massive activation of primordial follicles when FOXO3A is depleted (Castrillon et al., 2003) and *vice*

versa, an inhibition of oocyte growth when FOXO3A is constitutively expressed (Liu et al., 2007). These studies indicate the important role of FOXO3A, its phosphorylation status and localization, in the initial recruitment of dormant follicles into the pool of actively growing follicles. Additionally, the Hippo pathway was shown to be involved in follicle activation *in vitro*. Upon fragmentation of the ovarian cortex, Hippo signaling is disrupted which allows YAP to act as co-activator in the nucleus. Transcriptional activation of downstream targets, such as CCN growth factors, promotes follicular activation and growth (Grosbois et al., 2018; Kawamura et al., 2013). In human, a combination of PI3K stimulation and Hippo disruption by ovarian cortex fragmentation was used for treatment of patients suffering from premature ovarian insufficiency which led to successful pregnancy and live birth (Kawamura et al., 2013).

In mice, the existence of sub-populations of primordial follicles was reported (Zheng et al., 2014) with the "first wave" being immediately activated postnatally after they are formed and possibly responsible for induction of puberty onset and early fertility. In contrast, the adult "second wave" follicles are being activated gradually and supply follicles throughout adulthood. Based on this, a potential model of two distinct waves of primordial follicles was hypothesized to also apply to humans. This might explain morphological differences in primordial follicle structures found in pre-pubertal ovaries as compared to adult ovaries. Anderson et al. described morphologically abnormal non-growing follicles present to a high extent in the pre-pubertal ovaries. As this population showed a limited capacity for *in vitro* growth and are reduced in pubertal and absent in adult ovaries, they may potentially represent the "first wave" follicles described in mice (Anderson et al., 2014).

Taken together, a primordial follicle formed during human embryo development has three potential fates postnatally: death through atresia, arrest in a dormant state mediated through repressive signals (e.g. PTEN, FOXO3A) or its transition into a growing state upon activating signals (KITL, growth factors) (Tingen et al., 2009). Even though a lot of progress has been made during the last couple of years, especially driven by mouse studies, in-depth studies on the molecular mechanisms underlying the fate determination of primordial follicles are clearly missing.

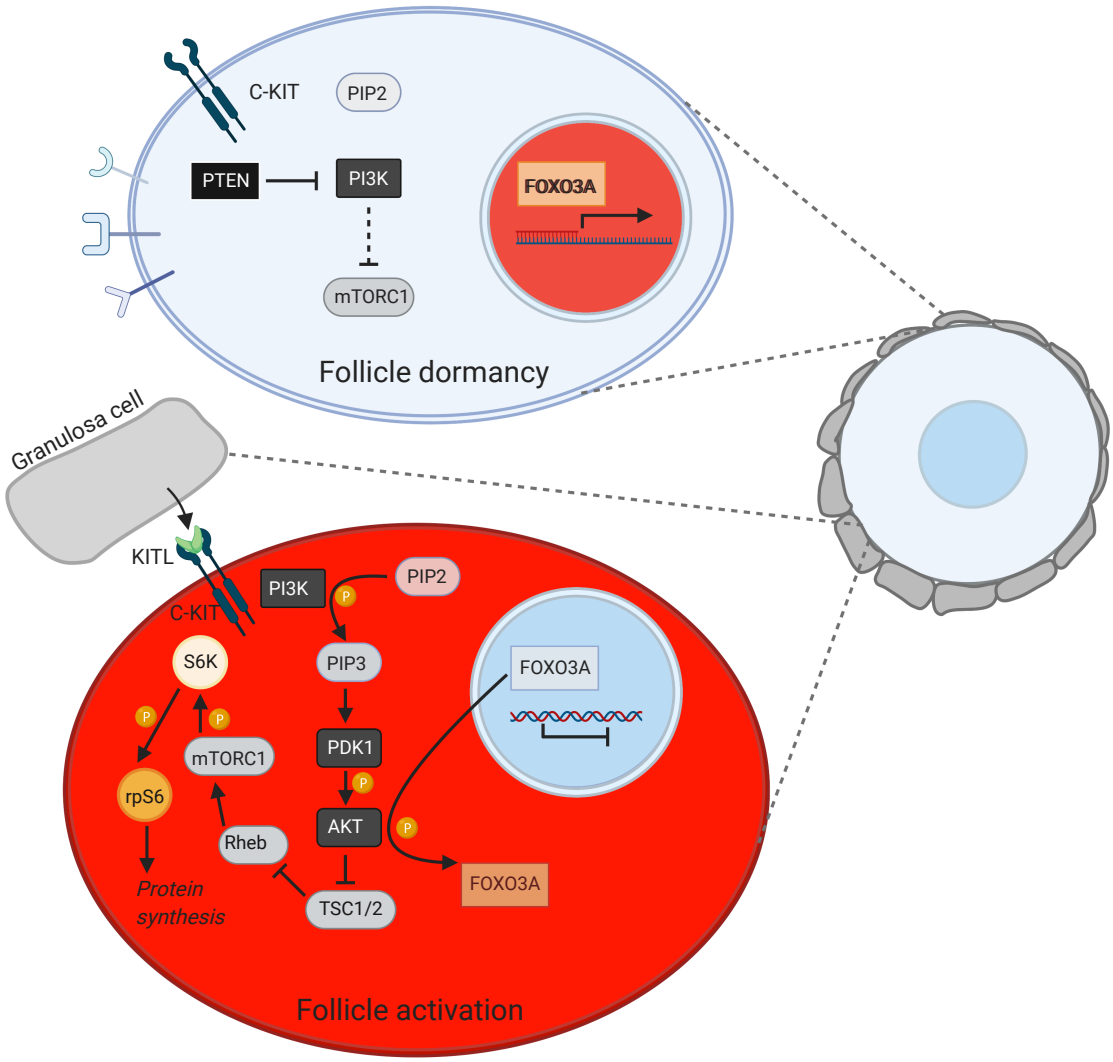

Fig.3 The PI3K/AKT signaling pathway is involved in primordial follicle activation. In the oocyte of a dormant follicle (upper diagram), PI3K is inhibited by PTEN which keeps FOXO3A unphosphorylated. Unphosphorylated FOXO3A is located in the nucleus where it can act as a transcriptional activator. Upon follicle activation (lower diagram), e.g. KITL from the granulosa cells binds to the C-KIT receptor on the oocyte, PI3K elicits a phosphorylation cascade. AKT kinase is phosphorylated which in turn phosphorylates FOXO3A. Phosphorylated FOXO3A translocates into the cytoplasm and cannot fulfil its function as a transcription factor any longer. In addition, TSC1/2 is inhibited and cannot act as inhibitor of Rheb. This activates mTORC1 which ultimately leads to activation of rpS6 and protein synthesis. Graphics was created using ©BioRender.

1.1.3 Ovarian aging and menopause

A woman enters menopause when the ovarian reserve is consumed to a level where around 1,000 follicles or less remain (Faddy et al., 1996; Faddy et al., 1992). On average, this is around the age of 50-51 years but was reported to depend on the size of the primordial follicle population at its peak during embryo development at 18-22 weeks of gestation (Wallace et al., 2010). A lower prenatal peak number of primordial follicles correlates with an early menopause onset and *vice versa*. Menopause is defined by amenorrhea for at least one year. On a physiological level, menopause reveals itself in the loss of the ability to conceive, a disturbed

thermoregulation ("hot flushes"), cardiovascular diseases and osteoporosis, among others (Davis et al., 2015). On an endocrine level, changes in the hormonal profile are observed, such as elevated FSH levels and reduced concentrations of estrogen and AMH. Serum AMH levels is a widely used biomarker to determine the size of the ovarian reserve and to predict the reproductive lifespan. Growing follicles (from secondary follicle stage onwards) secrete AMH and the growing follicle population was suggested to also be reflective of the non-growing follicle population, hence the ovarian reserve. After a peak of serum AMH levels around 24-25 years, levels decline with age (Kelsey et al., 2011). Ovarian aging was also found to correlate with an increasing collagen deposition in the ovarian stroma (Motta et al., 2002; Ouni et al., 2020). While elastic fibers decrease, the more stiff collagen fiber content increases and was suggested as contributing factor to menopause onset (Ouni et al., 2020). On a molecular level, changes in the ovary proceeding menopause onset are less known. However, a recent study on ovary aging in non-human primates suggested oxidative stress as a key player (Wang et al., 2020). The subsequent analysis of human granulosa cells suggested similar mechanisms at play during the process of human ovarian aging: granulosa cells isolated from follicular fluid showed an aging associated decrease in antioxidant activity and an increase in oxidative stress induced damage (Wang et al., 2020).

Age is a natural cause of menopause. However, early onset of menopause can be caused by premature ovarian insufficiency. The origins of premature ovarian insufficiency are manifold, including diabetes mellitus, autoimmune disorders, polycystic ovarian syndrome and chemical insults like smoking and anti-cancer treatment.

1.2 FERTILITY RELATED EFFECTS OF ANTI-CANCER THERAPY

Anti-cancer treatment improves constantly by early diagnoses and availability of more selective therapies, leading to a drastically increasing survival rate of patients (Siegel et al., 2017; Trama et al., 2019). Chemo- and radiotherapy damages ovarian follicles, with alkylating (e.g. cyclophosphamide and busulfan) and alkylator-like chemotherapy agents (e.g. cisplatin) being considered most detrimental (Morgan et al., 2012; Oktem et al., 2008) and recent studies show that adult patients cured from cancer of all types are 38% less likely to conceive (Anderson et al., 2018). Classically, rapidly proliferating cells (such as granulosa cells in a growing follicle) are targeted by cell-cycle specific chemotherapy drugs, such as antimetabolites (Yuksel et al., 2015), causing DNA adducts and DNA damage (see Fig.4). In contrast, many alkylating agents and anthracyclines used for chemotherapy treatment, such as cyclophosphamide and doxorubicin, respectively, are cell-cycle unspecific and hence, can affect primordial follicles in their dormant state (see Fig.4). Either way, the induction of DNA damage can ultimately lead to double strand breaks (DSBs) which initiates a so-called DNA damage response mechanism within the cell which – in its most deadly form – triggers cell

apoptosis. DSBs are also the most common form of DNA damage induced by irradiation (Thompson, 2012).

As chemotherapy treatment most often combines a plethora of different drugs, the risk of infertility is usually estimated based on the type of malignant disease and its associated treatment regimen (Wallace et al., 2005). For example, chemotherapy and radiation treatments of Hodgkin's disease as well as conditioning therapies for hematopoietic stem cell transplantation (HSCT) patients are associated with a very high risk (>80%) of subfertility after successful treatment through premature ovarian insufficiency at all ages (Meirow et al., 2001; Wallace et al., 2005; Warne et al., 1973). Premature ovarian insufficiency patients show the above mentioned menopausal symptoms at ages younger than 40 years, including an exhausted ovarian reserve, decreased pregnancy rate and hormonal imbalances, such as high serum levels of FSH, low levels of estrogens and decreased serum levels of AMH (Green et al., 2009; van Dorp et al., 2016; van Kasteren et al., 1999).
Adverse effects of chemotherapy on ovaries was reported to increase with age. The older the patient – hence, the smaller the remaining ovarian reserve – at the time of treatment, the more likely they will experience ovarian failure afterwards (Meirow et al., 2001).

Despite great progress in the field, the molecular mechanisms involved in anti-cancer treatment-triggered depletion of the ovarian reserve and its downstream effect on hormonal imbalances have not yet been fully unraveled. Potential targets of chemotherapy treatment within the ovarian follicle are both oocyte and granulosa cells as well as the stromal compartment including the vascular system of the ovary. Research in this field has mainly focused on the effect of anti-cancer treatment on ovarian follicles and currently suggests two main mechanisms, addressed in more detail below: "burnout" of the ovarian reserve through an over-activation of primordial follicles and follicle death induced by DNA damage.

1.2.1 "Burnout hypothesis"

The detrimental effect of chemotherapy on fertility is partly caused by destruction of the somatic cells of growing follicles. Proliferating, somatic ovarian cells are the first target of chemotherapy agents. Theca cells and granulosa cells are among the most active cell types in the ovary. As discussed earlier and depicted in Fig.2, these cells play a major role in the production of steroid hormones and growth factors in the ovary and once undergoing chemotherapy-induced apoptosis, the steroidogenic pathway and signaling network *via* local growth factors, e.g. AMH, is affected (Cohen, 2008; Yuksel et al., 2015). AMH is expressed in granulosa cells of growing follicles and inhibits initial follicle recruitment *via* negative feedback (Dumont et al., 2015).

This rationale of chemotherapy induced disruption of negative feedback signaling in the ovary led Prof. Meirow´s group to formulate the "burnout hypothesis" (Kalich-Philosoph et al., 2013; Meirow et al., 2010). This group studied the effect of cyclophosphamide in mice and their hypothesis also resonated with researchers studying chemotherapy effects on the bovine (Gavish et al., 2015) and human ovary (Gavish et al., 2015; Lande et al., 2017). Proliferating cells, including granulosa cells of growing follicles, are destroyed by chemotherapy (Lopez et al., 2004) which leads to an activation of dormant follicles due to the missing negative cues, such as AMH (Durlinger et al., 2002). Prof. Meirow's group observed this increased follicle activation through PI3K/AKT signaling and concluded that the ovarian reserve burns out prematurely due to an increased recruitment of primordial follicles into the pool of growing follicles (Kalich-Philosoph et al., 2013) (see Fig.4).

1.2.2 DNA damage induced follicle death

DSBs are another phenomenon through which chemotherapy-induced damage of ovarian follicles can occur (see Fig.4). DSBs introduced by chemotherapy agents can be handled in two ways: the oocyte resolves those DSBs by either repair or death. Primordial follicles in the mouse and human ovary were shown to be able to repair DSBs to some extent (Winship et al., 2018). However, when damage cannot be repaired, oocyte death is mediated *via* apoptosis. It was shown in a xenograft model that a single dose of cyclophosphamide results in significant loss of primordial follicles in human ovarian tissue through the mechanism of apoptosis (Oktem et al., 2007a).

TAp63 was identified as the key mediator of apoptosis in ovarian follicles upon anti-cancer treatment (Kerr et al., 2012). TAp63 is a p53 family member transcription factor, highly conserved in mammals and specifically expressed in the female germline (Gonfloni et al., 2009; Levine et al., 2011; Soleimani et al., 2011a; Suh et al., 2006). TAp63 activates the pro-apoptotic protein p53 upregulated modulator of apoptosis (PUMA) which mediates induction of apoptosis. The mechanism of apoptosis induction in follicles upon chemotherapy treatment was elegantly proven in mice (Nguyen et al., 2018). Chemotherapy treated $Puma^{-/-}$ mice showed full retention of primordial follicles by repair of induced DSBs, while primordial follicle reserve was reduced down to 4% in chemotherapy treated wildtype mice. As rescued primordial follicles could contribute to healthy reproduction, the authors suggested the inhibition of PUMA as ovary-protective treatment in women undergoing chemotherapy to preserve the ovarian reserve (Nguyen et al., 2018).

Along those lines, LH was recently suggested as another ovo-protective agent as it was found to preserve the ovarian reserve in pre-pubertal mice undergoing chemotherapy treatment using cisplatin (Rossi et al., 2017). The mechanism behind this protective effect is potentially mediated through the observed increase in anti-apoptotic signals generated by LHR expressing

somatic cells. Anti-apoptotic signals led to lower levels of TAp63 and an overall nudge towards DSB repair rather than apoptosis of the ovarian reserve follicles. However, it is not fully understood yet which somatic cells would mediate this anti-apoptotic effect *via* LHR in the prepubertal ovary and warrants deeper investigation.

Bedoschi et al. carefully reviewed the data published on chemotherapy induced follicle loss and concluded that the data available to support the "burnout hypothesis" are insufficient yet while "…the evidence for DNA damage induced follicle death is highly convincing …" (Bedoschi et al., 2016).

1.2.3 Chemotherapy effect on the stromal compartment of the ovary

Studies show that ovarian follicles are also likely to be damaged more indirectly *via* detrimental effects of anti-cancer treatment on the stromal compartment in the ovary. The ovarian stroma is highly vascularized by several factors including blood and nutrient supply to growing follicles. Several histological studies on ovarian tissue sections from pre- and post-pubertal cancer patients reported the increased appearance of fibrosis and an altered structure of the vasculature. They describe a higher prevalence of fibrotic areas as well as vessel thickening (Marcello et al., 1990; Meirow et al., 2007; Nicosia et al., 1985), lower levels of neo-angiogenesis along with lower density of mature blood vessels (Soleimani et al., 2011a), possibly explaining the lower blood flow observed in chemotherapy-exposed ovarian tissue (Ben-Aharon et al., 2012) (see Fig.4). Although it has long been believed that dormant, primordial follicles – in contrast to growing follicles – do not depend on vascularization, an inverse correlation was shown between vascular density and the percentage of atretic primordial follicles in xeno-transplanted human ovarian tissue (Soleimani et al., 2011b). In other words, (neo-)vascularization seems to be required for the survival of even dormant follicles. However, to this date, the connection of altered stroma/vasculature and ovarian follicle quality has not been studied on a molecular level. And it remains speculative how exactly a damaged ovarian stroma with increased fibrosis and altered vasculature would affect the ovarian follicle reserve and/or ovarian follicle development.

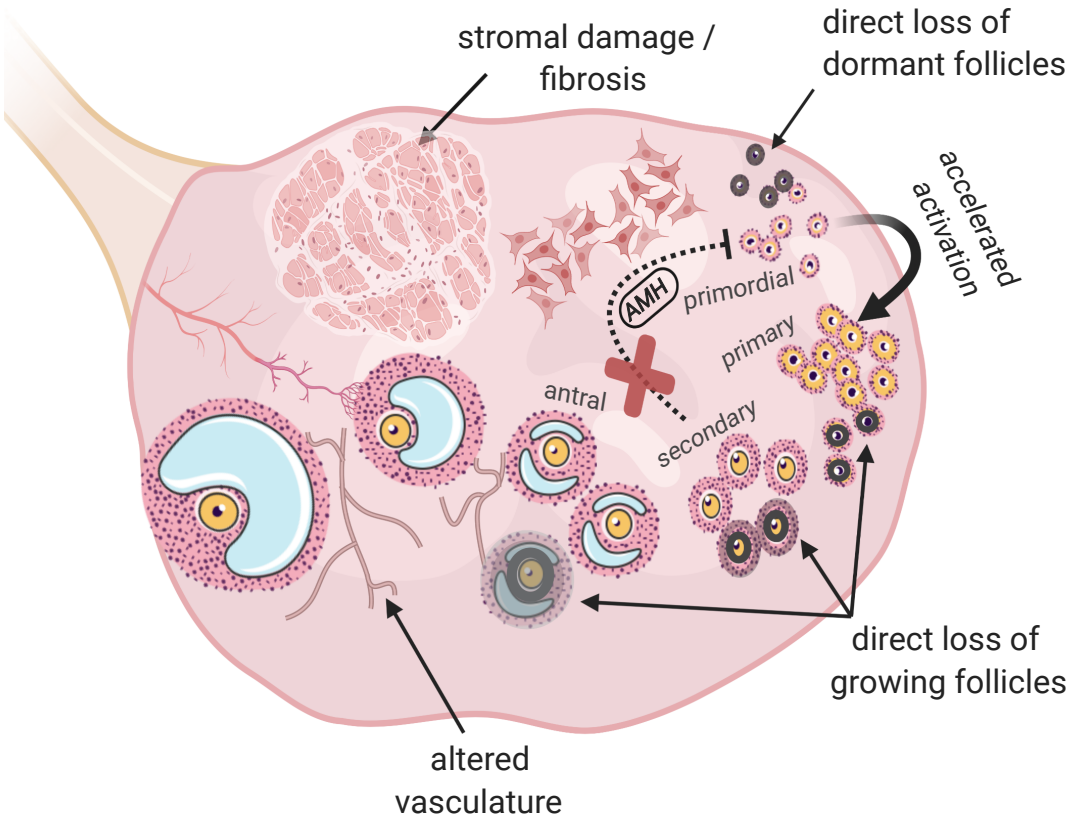

Fig.4. Potential effects of chemotherapy on the human ovary. Chemotherapy treatment was reported to impair the ovarian follicles *via* different routes: directly *via* induction of DNA damage into dormant and growing follicles and/or indirectly *via* a premature depletion of the ovarian reserve, an altered vasculature, and fibrotic lesions. Premature depletion of the ovarian reserve based on the "burnout hypothesis" suggests that AMH-mediated inhibition of primordial follicles goes missing upon apoptosis of growing follicles, thereby accelerating primordial follicle activation and depletion of the ovarian reserve. Graphics was created using ©BioRender.

1.2.4 Fertility treatment options for cancer survivors

Ultimately, unravelling the mechanisms behind chemotherapy-induced follicle loss serves to develop a strategy to prevent the depletion of the ovarian reserve and maintain the integrity and quality of follicles in order to preserve future fertility of the patient. So far, the only two treatment options for patients at high risk of future infertility due to treatments are fertility preservation through cryopreservation of embryos/oocytes or ovarian tissue. Embryo and oocyte cryopreservation are established interventions but can only be offered to post-pubertal and adult patients as it requires the availability of mature oocytes. The cryopreserved embryo or oocyte can be thawed for standard fertility treatments through IVF and embryo transfer after recovery of the patient (Donnez et al., 2017).

For pre-pubertal cancer patients in very high risk of future infertility and patients that cannot delay chemotherapy onset, autologous ovarian tissue cryopreservation is considered the only alternative. In theory, one can preserve thousands of primordial follicles at once with this

procedure. Transplantation of frozen/thawed ovarian tissue (Ovarian Tissue Transplantation, OTT) has been proven successful for adult women and more than 130 live births have been reported up to date (Donnez et al., 2017). Cohort studies showed that in adults, OTT of ovarian tissue exposed to chemotherapy agents prior to cryopreservation results in similar rates of endocrine function, pregnancy and live births when compared to OTT with unexposed ovarian tissue (Meirow et al., 2016; Poirot et al., 2019). Case reports documented that OTT following ovarian tissue cryopreservation in pediatric patients was used to induce puberty in two patients (Ernst et al., 2013; Poirot et al., 2012) and successful pregnancy could be achieved after transplantation of cryopreserved ovarian tissue harvested at an age of thirteen and nine years, respectively (Demeestere et al., 2015; Matthews et al., 2018). It is uncertain whether these two patients classified as pre-pubertal at the time of ovarian tissue cryopreservation. Even though the patients were pre-menarche, hormone levels and breast development indicated puberty onset in the case of the thirteen-year-old girl and were not documented for the nine-year-old girl.

Since last year, OTT in pediatric patients is no longer considered experimental by the American Society for Reproductive Medicine (ASRM, 2019). However, as highlighted by the Pediatric Initiative Network in a response letter, ovarian tissue cryopreservation research needs to be continued as risks and benefits are not fully understood in the pediatric population (Nahata et al., 2020). Valuable concerns regarding follicle quality in transplanted tissue of young patients have been raised. In young patients, it was suggested that a younger age (children *versus* young adolescents) protects against chemotherapy-induced infertility due to the bigger ovarian reserve (El Issaoui et al., 2016). However, in human *in vitro* studies, morphological abnormalities and reduced developmental potential was reported for primordial follicles (Anderson et al., 2014; Asadi Azarbaijani et al., 2015) and oocytes of primordial follicles (Abir et al., 2016) of pre-pubertal ovaries, independent of chemotherapy treatment. This could suggest that follicles from young girls are more fragile compared to adults and would support the hypothesis of two waves of primordial follicles in the human ovary (Anderson et al., 2014). Ideally, ovarian tissue cryopreservation should be done before chemotherapy onset due to the gonadotoxic effects elaborated in the previous sections. However, this might not be feasible in cases when immediate treatment onset is necessary, when first-line chemotherapy appears ineffective and more intensive treatment is required or when patients relapse (Wallace et al., 2016). Selection criteria of pre-pubertal patients to be offered ovarian tissue cryopreservation are not fully harmonized among different centers. According to the recommendations established by the Nordic Society for Pediatric Hematology and Oncology (NOPHO 2013), in the Nordic countries (Sweden, Finland, Norway, Denmark and Iceland), only pre-pubertal patients who are receiving anti-cancer treatment associated with a very high risk of POI can be referred to fertility preservation through ovarian tissue cryopreservation. Very-high risk treatments include conditioning therapies for allogenic or autologous hematopoietic stem cell transplantation or radiotherapy in the ovary field. However, as mentioned above, patients might

only fall into the very high-risk group after initial ineffective first-line chemotherapy treatment with agents of a lower risk group. In these cases, ovarian tissue cryopreservation is performed with ovarian tissue exposed to (first-line) chemotherapy agents.

Of note, another concern is the re-introduction of malignant cancer cells upon transplantation of cryopreserved ovarian tissue harvested prior to chemotherapy treatment. This particularly concerns certain cancer types, such as leukemia (Dolmans et al., 2010). Therefore, only patients with a low risk of ovarian metastatic cells are considered for OTT (Dolmans et al., 2013), further restricting the patient group that could benefit from current fertility preservation options. Although latest advances in ovarian tissue cryopreservation have improved the success rate of female fertility preservation tremendously (Anderson et al., 2017; Oktay et al., 2018), data on the safety and efficacy after ovarian tissue cryopreservation are still limited. Furthermore, molecular mechanisms of follicle loss through cytotoxic agents are still not fully understood and remain to be identified.

ABVD-type chemotherapy is commonly used to treat Hodgkin's lymphoma and is composed of the chemotherapeutic drugs adriamycin, bleomycin, vinblastine and dacarbazine. ABVD treatment is considered low risk in terms of infertility (Hodgson et al., 2007). Quite the contrary, following this treatment, a surprising increase in the amount of dormant, non-growing follicles was observed in the tissue of pediatric and young adult patients when compared with age-matched predicted values (McLaughlin et al., 2017). The underlying mechanism of this observed phenomena is not understood. One proposed explanation is the activation of tissue resident stem cells which might have the capacity to differentiate into new oocytes, thereby regenerating the pool of dormant follicles upon this specific chemotherapy treatment (McLaughlin et al., 2017).

1.3 OOGONIAL STEM CELLS IN THE ADULT OVARY

The dogma of a fixed ovarian reserve in mammals has long been debated. Latest reports in ovarian research brought up the possibility of an existing germline stem cell population (also and from now on termed oogonial stem cells, OSCs) in the adult mammalian ovary (Johnson et al., 2004; White et al., 2012; Zou et al., 2009). OSCs were reported to have the potential to differentiate into oocytes and hence, to hold great potential for the treatment of infertility or chemotherapy-induced premature ovarian insufficiency.

It is well-known that some non-mammalian species harbor germline stem cells in the adult ovary. In invertebrates, e.g. the fly *Drosophila* and the nematode *Caenorhabditis elegans* (*C. elegans*), oocytes are consistently being produced through germline stem cell differentiation in the adult ovary. In *Drosophila*, germline stem cells are sitting at the tip of each ovary (termed ovariole) in a niche comprised of three somatic supporter cell types. Germline stem cells divide

asymmetrically assuring that the germline niche does not exceed the size of approximately two germline stem cells (Spradling et al., 2011). In *C. elegans*, the Distal Tip Cell located at the distal tip of the gonads is maintaining a stable pool of germline stem cells that ensures the stable production of gametes in the adult worm (Kimble et al., 2008). However, non-mammalian vertebrate models, such as zebrafish (*Danio rerio*) and frog (*Xenopus leavis*), are less studied and no solid conclusions regarding the presence of germline stem cells in their postnatal ovary have been reached yet.

In mammalian species, the dogma of a fixed ovarian reserve and hence the absence of female germline stem cells in the postnatal ovary was challenged in 2004 when the research group of Prof. Tilly reported the existence of OSCs in adult mouse ovary (Johnson et al., 2004) and eight years later in adult human ovary (White et al., 2012). In the initial mouse study (Johnson et al., 2004), the authors investigated follicle degeneration from birth until entry into reproductive senescence. They found that the ovarian reserve should be depleted much earlier than reported before (Faddy et al., 1987) when taking into account the observed rate of follicle degeneration and concluded that neo-oogenesis takes place in the postnatal mouse ovary. Indeed, when evaluating the ovary morphologically, they found large ovoid cells in the surface epithelium that resembled germ cells and expressed the germline marker DDX4 (DEAD-box polypeptide 4) at the protein level and the proliferation marker BrdU (5-bromodeoxyuridine). Those large ovoid cells were suggested to be OSCs. In a follow-up study, they documented that OSCs might originate from bone marrow and peripheral blood (Johnson et al., 2005). This was hypothesized from the fact that PGCs and hematopoietic cells originate in the same region during embryogenesis (Ratajczak, 2017).
Subsequently, Tilly's group established a protocol to study OSCs from mouse ovaries (Zou et al., 2009). For this, OSCs were isolated by magnetic-activated cell sorting (MACS) using a specific antibody directed against the C-terminal domain of the germ cell marker DDX4 (Fig.5). OSCs from adult mouse ovaries could be isolated and cultured long-term, and they gave rise to viable offspring when transplanted into ovaries of infertile mice (Zou et al., 2009).

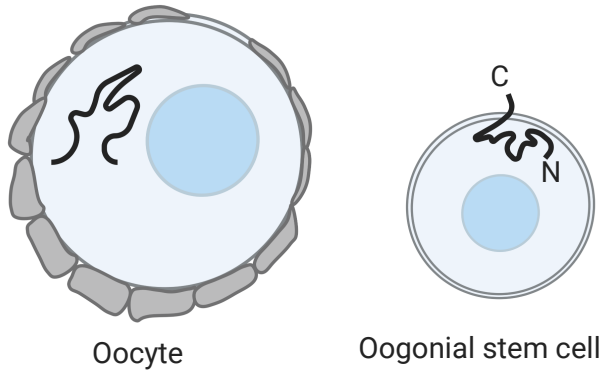

Fig.5. Cellular localization of DDX4 in the mouse and human ovary based on OSC hypothesis. While the oocyte is known to express DDX4 protein in the cytoplasm throughout all developmental stages (Castrillon et al., 2000), the C-terminal domain of DDX4 in OSCs was reported to have an extracellular epitope (White et al., 2012). Graphics was created using ©BioRender.

This protocol using MACS was modified to the usage of fluorescence-activated cell sorting (FACS) when OSCs in mouse and human adult ovary were reported, yielding in a higher purity of the population of interest (White et al., 2012). This small population of DDX4 antibody (Ab)-positive cells (around 1.7% in human and 1.5% in mouse) was shown to express germ cell markers (PRDM1 and C-KIT) on a transcriptional level and to grow in size when cultured *in vitro*. When these putative human OSCs were GFP-labelled and injected into human ovarian cortex and further xeno-transplanted into mouse ovaries, big green fluorescing cells (potentially human oocytes) were found being surrounded by smaller non-fluorescent cells (potentially mouse granulosa cells) after one week (Fig.6). Based on these findings, the authors concluded that cells isolated with the DDX4 antibody comprise germline stem cell characteristics with the potential to differentiate into oocytes *in vivo*.

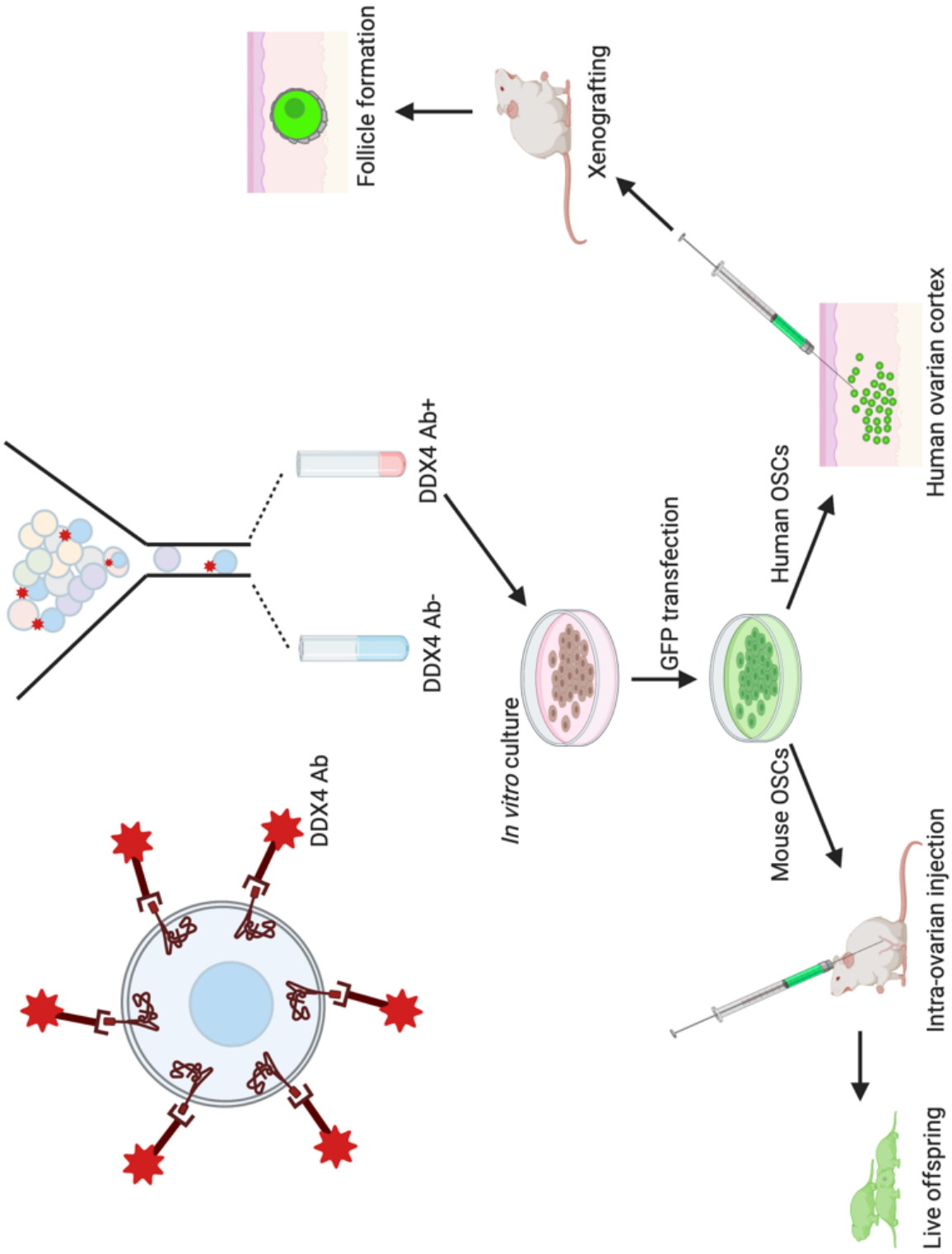

Fig.6. Published isolation protocol of OSCs (Woods et al., 2013). DDX4 Ab is used for sorting of DDX4 Ab-positive and Ab-negative populations. Sorted DDX4 Ab-positive cells were cultured under OSC conditions *in vitro* and transduced with GFP vector. GFP-expressing mouse OSCs were injected into mouse ovaries and viable, GFP-positive offspring was generated. GFP-expressing human OSCs were injected into human ovarian cortex and xeno-grafted under the skin of immunodeficient adult mice. Formation and maturation of GFP-positive oocytes was observed after one week of transplantation. Graphics based on Woods and Tilly 2013 with permission and created using ©BioRender.

1.3.1 The role of DDX4 in the germ cell lineage

DDX4 as a germline marker is a universal and highly conserved member of the DEAD box protein family and, just like DAZL, an RNA-binding protein. While their specific functions are not fully understood yet, both are known to play a role in protein stabilization and localization during PGC formation and considered crucial drivers of germ cell determination (Nicholls et al., 2019; Smorag et al., 2014). *In vitro* studies on human PGCs and murine *in vivo* and *in vitro* studies indicate that DAZL is operating upstream of DDX4, reporting an upregulation of DDX4 upon DAZL overexpression – but not *vice versa* (Canovas et al., 2017) – probably due to translational regulation of DDX4 through DAZL (Reynolds et al., 2005).

DDX4 protein is an ATP-dependent RNA helicase (Castrillon et al., 2000) and based on its protein conformation as well as on experimental and *in silico* data, DDX4 is considered to be a cytoplasmic protein (Zarate-Garcia et al., 2016). Experimental data however are contradictive with studies reporting DDX4 localization to be restricted to the cytoplasm (Fujiwara et al., 1994; Nott et al., 2015) but also to be found on surface of transfected human embryonic kidney (HEK) 293T cells (Clarkson et al., 2019). It's been believed to be exclusively expressed in the germline from around week 7 of human gestation onwards and maintained throughout development and differentiation of oogonia into oocytes of primordial follicles (Castrillon et al., 2000; Dorfman et al., 1999) (Fig.5). Similarly, in fetal testis, DDX4 can be detected intra-cytoplasmically in spermatocytes and spermatogonia.

DDX4 was first identified in *Drosophila melanogaster* (named Vasa in *Drosophila*) where it is regulating translation of mRNA expression during germ cell development (Hay et al., 1988). Vasa null mutations were reported to lead to female – but not male – sterility in *Drosophila*, whereas homozygous mutations, with partially remaining Vasa function, allow formation of embryos which lack germ cells (Schupbach et al., 1986).
In contrast, mice show a pronounced effect on male fertility when DDX4 (also called Mouse vasa homolog, MvH) is mutated: male homozygous mutants are sterile, while female mice don't show any reproductive abnormalities and remain fertile (Tanaka et al., 2000).
Although the crucial role of DDX4 during PGC determination would suggest it, so far, no findings have yet been reported that would link DDX4 mutations to fertility-related problems in humans.

1.3.2 Attempts to validate the existence of OSCs in human adult ovaries

As all potential shifts in paradigm, Tilly´s work was highly criticized. Follow-up research on putative OSCs in mouse and human postnatal ovary by other groups presented conflicting results. In this section, I will focus on studies investigating putative OSCs in adult human ovary only.

The method used to isolate putative OSCs heavily relies on a polyclonal Ab directed against the C-terminal domain of DDX4 protein (Woods et al., 2013). As mentioned above, DDX4 protein is considered to be expressed in the cell cytoplasm *in vivo*. This would not allow an isolation of viable OSCs by FACS which is a technology that requires extracellular proteins as targets. However, some studies suggest that OSCs display a surface epitope of DDX4 C-terminus that can be used to target this cell population (Woods et al., 2013) (Fig.5).

Reproducibility of the data reported by Prof. Tilly´s group has been challenging. Although all groups, including our laboratory, demonstrate the successful isolation of cells from the human ovary using the same DDX4 Ab (ab13840 from Abcam), their abundancy is varying among different studies, ranging from 2.5 – 24% (Hernandez et al., 2015; Zarate-Garcia et al., 2016; Zhang et al., 2015) as compared to 1.7% initially documented by Tilly (White et al., 2012). In addition, while some groups were able to detect *DDX4* mRNA in freshly isolated DDX4 Ab-positive cells (Clarkson et al., 2018; Ding et al., 2016b; White et al., 2012), others were not – neither by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Hernandez et al., 2015; Zarate-Garcia et al., 2016; Zhang et al., 2015), nor by single-cell mRNA sequencing (scRNA-seq) (Zhang et al., 2015). Mass-spectrometry analysis did not detect DDX4 protein either, although cells were isolated based on detection of DDX4 by DDX4 Ab using FACS (Hernandez et al., 2015). The authors of these studies concluded that DDX4 Ab might bind to unspecific surface epitopes and that the DDX4 Ab-positive cells do not actually express *DDX4* (Hernandez et al., 2015; Zarate-Garcia et al., 2016; Zhang et al., 2015). Boellaard et al. specifically investigated whether a positive signal for DDX4 by immunostaining is sufficient to identify germ cells in male urogenital tissue and concluded that immunocytochemistry with different DDX4 Abs (among those the Ab used in the OSC isolation protocol) is unspecific compared to mRNA evaluation. Hence, only transcriptome-based results are indicative of DDX4 expression and should be used for germ cell detection (Boellaard et al., 2017).

Many lines of evidence suggest that the DDX4 Ab-based OSC isolation protocol is not specific and may yield a heterogeneous mixture of cell types. This was recently confirmed by Prof. Telfer's group, who investigated molecular characteristics of DDX4 Ab-positive cells from human ovaries isolated with the DDX4 Ab-based FACS protocol. Three distinct DDX4 Ab-positive populations in ovarian cortex cell suspension were found that differ in size, aldehyde dehydrogenase 1 (ALDH1) activity, and a different expression pattern of DDX4 splice variants and of DAZL. Furthermore, they show that this heterogeneous DDX4 Ab-positive cell population can form follicle-like structures in a fetal somatic cell environment (Clarkson et al., 2018).

Besides the already mentioned widely criticized use of DDX4 Ab for OSC isolation, the OSC isolation protocol comes with several technical challenges. Issues such as ovarian tissue source,

handling, digestion into single cells and Ab labeling can differ slightly among different laboratories. Gating strategies for FACS based isolation of OSCs are rarely reported but is a crucial step prone to inconsistencies when cell populations are sorted by different operators. As frequently brought up in discussions around OSCs, all this can have a great impact on cell yield, viability and detection of small sub-populations (Woods et al., 2013, 2015b). And finally, lot-to-lot differences in enzymes and the polyclonal DDX4 antibody recommended for OSC isolation have been reported to impact the detection and purity of the final product (Woods et al., 2013). Of note, centrifugation speed of the obtained ovarian single cell suspension was also deemed crucial when isolating stem cell populations from ovarian tissue (Bhartiya et al., 2020). Slight methodological divergence between different groups might hence explain the inconsistencies found in literature with regards to nature and characteristics of isolated DDX4 Ab-positive cells. Clearly, the published protocols for isolation of OSCs must contain more detail in order to ensure successful isolation of these cells across laboratories.

In addition to the reports of putative OSCs in adult human ovarian cortex, some research groups have postulated the existence of a cell population with pluripotency potential in the adult human ovarian surface epithelium. Those so-called very small embryonic-like cells (VSELCs) were found to express the pluripotency markers POU5F1, SSEA-4, NANOG, SOX2 (shown by qPCR and immunocytochemistry) as well as DDX4 using immunocytochemistry (Parte et al., 2011; Stimpfel et al., 2013; Virant-Klun et al., 2013b). It is unknown yet whether VSELCs and OSCs co-exist in the human ovary or if – as suggested by the authors of VSELC studies (Bhartiya et al., 2018; Virant-Klun et al., 2013a) – VSELCs might be the precursor cells giving rise to OSCs.

1.3.3 Clinical relevance of OSCs

The discovery of putative OSCs in the adult human ovary has reached the media at an early stage and raised big hopes among clinicians and their female patients facing infertility. In 2011, the company OvaScience^{SM} was founded that offers fertility treatments based on DDX4 Ab-positive putative OSCs at selected clinics. The first treatment launched, called AUGMENT^{SM} (Autologous Germline Mitochondrial Energy Transfer, Fig.7), involves the isolation of DDX4 Ab-positive cells from a biopsied sample of ovarian cortex of the patient. The isolated cells are cryopreserved and when needed, thawed to extract their mitochondria. These autologous mitochondria are injected along with one sperm cell into the patient's own oocytes in order to supply additional energy that might improve pregnancy rate after *in vitro* fertilization, IVF (Woods et al., 2015a) (Fig.7).

In 2015, the first report has been published that documented the outcomes of AUGMENT^{SM} treatment in patients at two clinics (Fakih, 2015). The study enrolled two cohorts of difficult-to-treat women in Dubai, UAE, and Toronto, Canada, respectively. In summary, the authors

showed an improved pregnancy rate when the AUGMENT^{SM} treatment was used as compared to conventional intracytoplasmic sperm injection, ICSI, explained by a better embryo morphology after fertilization of the "boosted" oocytes. However, this study has several shortcomings. For example, the doctors were neither blinded nor was oocyte allocation to experimental groups randomized. In addition, the authors have stock ownership in OvaScience^{SM}. A second study investigated the effect of AUGMENT^{SM} treatment in women in Turkey with at least two previously unsuccessful IVF cycles and found a significant improvement in fertilization rates as compared to the previously unsuccessful IVF cycles (Oktay et al., 2015). Unfortunately, no controls were included here, and conclusions have to be taken cautiously. In 2018, Labarta and colleagues investigated the AUGMENT^{SM} treatment in a randomized, controlled, triple-blinded, experimental study at the Valencian Institute of Infertility, Spain (Labarta et al., 2019). Up to date, this represents the most thorough clinical study of AUGMENT^{SM} treatment in humans. Here, the authors received DDX4 Ab-positive cells from OvaScience^{SM} to perform AUGMENT^{SM} treatment in women with at least one previously unsuccessful IVF cycle. As a control, conventional ICSI was performed on half of the woman's oocytes retrieved after ovarian stimulation. In summary, the authors could not find any statistical difference in terms of fertilization rate, blastocyst formation rate and cumulative live birth rates when compared to the control group.

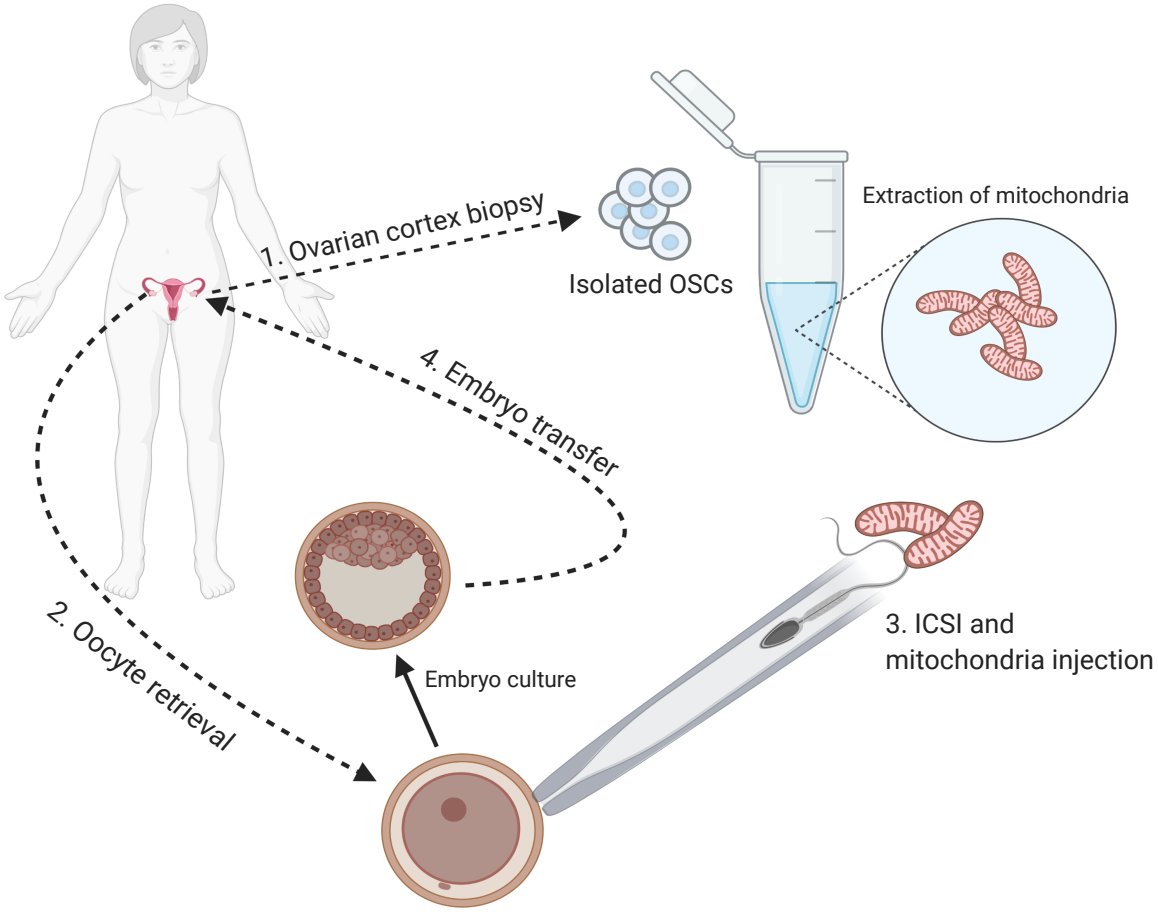

Fig.7. AUGMENT^{SM} treatment as infertility therapy. In women having difficulties to conceive due to a decreased oocyte quality, ovarian cortex biopsy is taken *via* laparoscopy. After isolation of OSCs, mitochondria are extracted. After oocyte retrieval, the woman's own oocyte is injected with the extracted mitochondria along with the sperm cell using ICSI. After *in vitro* maturation, the embryo is transferred back into the woman. Graphics was created using ©BioRender.

Altogether, a congruent study proving the existence of OSCs in human adult ovary that have the potential to develop into competent oocytes is still missing. Although considering putative OSCs for a therapeutic approach is tempting, for example in patients facing premature ovarian insufficiency, further research is clearly needed to evaluate the physiological role and clinical relevance of DDX4 Ab-positive ovarian cells that are not yet widely accepted in the scientific community (Vanni et al., 2017).

2 AIMS

The aims of this thesis are to determine the effect of chemotherapy on the human ovary, to investigate the cell type composition of the adult human ovarian cortex on a single-cell level and to characterize putative OSCs.

The specific aims of the two projects were:

Study I: to assess the impact of first-line chemotherapy on the human ovarian cortex from pediatric and young adult cancer patients using tissue samples from fertility preservation programs.

Study II: to map the different cell types within the adult human ovarian cortex with special focus on previously reported OSCs using advanced single-cell technologies as well as to investigate a possible existence of germline-like stem cells based on transcriptome profile.

3 MATERIAL AND METHODS

This section summarizes the key methods used during my PhD studies. All material and methods used in my studies are described in detail in the respective studies.

3.1 ETHICS AND OVARIAN TISSUE DONORS

In study I, ovarian tissue stored from a total of 43 pediatric and young adult patients for fertility preservation purposes was donated after informed written consent. Cryopreservation of ovarian tissue of pediatric patients was part of a research project approved by the Ethics Committee of Helsinki University Central Hospital (license number 340/13/03/03/2015) and was performed at Helsinki University Central Hospital (Finland). Informed written consent was given by the guardians of pediatric patients and patients themselves if age appropriate. Adult patients underwent ovarian tissue cryopreservation as part of a fertility preservation program at Tampere (Finland) and Oslo (Norway) University Hospitals and gave informed written consent for the quality control of ovarian tissue, including morphological analysis and *in vitro* culture. In accordance with the Medical Research Act (488/1999) and the Act on the Medical Use of Human Organs, Tissues and Cells (101/2001), no further ethical permits were needed.

In study II, ovarian tissue usage for research was approved by Stockholm Region Ethical Review Board (Dnr. 2010/549–31/2, Dnr. 2015/798–31/2) and included a total of 21 patients. Small biopsies (5x5x1 mm) were taken from cesarean section (C-sec) patients' ovarian cortex as well as whole ovaries from gender reassignment patients (GRPs) after informed written consent. All samples were collected at Karolinska University Hospital (Sweden).

3.2 OVARIAN TISSUE

3.2.1 Cryopreservation and thawing of adult ovarian cortex

In study I, previously cryopreserved, thawed and fixed samples were used.

In study II, tissues were collected, cryopreserved and thawed by our laboratory. GRP ovaries were trimmed using scalpels, the medulla part was removed and the remaining cortex of a thickness of approximately 1 mm was cut into pieces. C-sec biopsies did not require trimming and were cut into pieces immediately.

In order to store ovarian tissue whilst ensuring the maintenance of the biological structure and function long-term, ovarian tissue is frozen in a controlled manner using cryo-protectants. In our laboratory, we are using Ethylene Glycol to protect tissue cells from freezing damage.

For vitrification, tissue was cut into small pieces of approximately 1x1x1 mm and placed into vitrification solution containing 40% Ethylene Glycol, Sucrose and 10 mg/mL human serum albumin (HSA). After two incubation steps of 2 min and 3 min, respectively, the tissue was placed into cryotubes and stored in liquid nitrogen. For thawing, cryotubes were warmed at 37 °C before tissue pieces were transferred to thawing solutions of increasing HSA and decreasing sucrose concentration for 2 min, 3 min and 5 min, respectively.

For slow-freezing, tissue was cut into pieces of approximately 10x10x1 mm. After a pre-equilibration step in slow-freezing medium containing 7.5% Ethylene Glycol, Sucrose and 10 mg/mL HSA for 30 min, max. two pieces were placed into one cryotube containing 1 mL of slow-freezing medium. Cryotubes were transferred into the controlled rate freezer programmed as follows: - 2 °C/min to – 9 °C, hold at – 9 °C for 5 min, manual seeding by briefly touching the outer wall of the cryotube with liquid nitrogen-soaked cotton pad, - 0.3 °C/min to – 40 °C, - 10 °C/min to – 140 °C. After completion, cryotubes were stored in liquid nitrogen. For thawing, cryotubes were warmed at 37 °C before pieces were transferred to three different thawing solutions containing 10 mg/mL HSA and decreasing concentrations of sucrose for 10 min, respectively.

3.2.2 Histological analysis

In study I, tissue was freshly fixed in paraformaldehyde (PFA) or frozen/thawed and fixed in Bouin's solution. In study II, tissue was freshly fixed in methanol-free formaldehyde. Fixed tissue was embedded in paraffin and cut into sections of 4 µm. For all subsequent staining methods on fixed tissue sections including immunohistochemistry and FISH, assays were performed on tissue sections de-paraffinized in Xylene and rehydrated through ethanol series. Sections were re-hydrated with dH2O and washed with Tris-buffered saline (TBS, pH 7.6).

For **morphological classification** of ovarian follicles in study I, Bouin's fixed ovarian cortex sections were stained with hematoxylin and eosin and scanned using a spectral scanner. After evaluation of inter-observer concordance of more than 90%, two observers counted half of the patient material. For this, every firth section was analyzed to avoid double counting of follicles. Only follicles with a visible oocyte nucleus were taken into consideration when categorized and measured in Pannoramic Viewer software. For categorization, a follicle was counted as "healthy" if the round follicle shape was preserved, no eosinophilic cytoplasm present, the granulosa cell layer(s) were in full contact with the oocyte and the oocyte nucleus was round, central and not pyknotic. If one of the above criteria did not apply, such as delocalized granulosa cells or a distorted oocyte nucleus shape, the follicle was counted as "atretic". For classification of follicles based on developmental stage, established morphological criteria were followed (Gougeon, 1996): oocytes of primordial follicles were surrounded by one layer

of flattened granulosa cells; intermediary follicles showed flattened and at least two cuboidal granulosa cells; follicles were classified as primary follicles if one full layer of cuboidal granulosa cells was seen; secondary follicles had more than one full layer of cuboidal granulosa cells (see also Fig.1C-G).

To determine the amount of **fibrotic areas** in ovarian tissue in study I, trichrome stain kit was performed on Bouin's fixed ovarian cortex sections. Firstly, to stain cell nuclei in black, tissue sections were incubated in Weigert's hematoxylin for 5 min. An incubation in Biebrich scarlet/acid fuchsin solution for 15 min stained cytoplasm and muscle fibers in red. Following an incubation in phosphomolybdic/phosphotungstic acid solution for 10 min, tissue collagen was detected by staining in aniline blue solution (5 min) and acetic acid solution (3 min). After dehydration in solutions with an increasing ethanol concentration, tissue was cleared, mounted and imaged using light microscopy and analyzed using ImageJ.

To detect **stromal cell death** in ovarian tissue in study I, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining assay was performed on PFA-fixed ovarian cortex sections. The sections were incubated with Proteinase K to remove protein followed by an incubation in TUNEL reaction mixture for 1 h at 37 °C. This mixture contains the enzyme terminal deoxynucleotide transferase (TdT). TdT catalyzes the attachment of fluorescently labeled deoxynucleotides to the 3'hydroxyl termini of DNA and thereby, enables the visualization of DNA double-strand breaks. As positive control, tissue sections were treated with DNase I prior to TUNEL reaction. As negative control, sections were incubated in reaction mixture without TdT enzyme. Images were analyzed using confocal microscopy and ImageJ.

3.2.3 Immunohistochemistry

For detection of protein expression in tissue in study I and II, immunohistochemistry on PFA-fixed ovarian cortex sections was performed. Antigen retrieval was done in sodium citrate buffer (pH 6) at 96 °C and unspecific binding was blocked using serum. Primary antibodies were incubated over night at 4 °C. After thorough tissue washing, fluorescently labeled secondary antibodies and DAPI as nuclear counterstain were applied for 1h (study I) or 30 min (study II) on room temperature. Tissue sections were washed, mounted and analyzed using confocal microscopy and ImageJ.

3.2.4 Fluorescence *in situ* Hybridization

RNA transcript can be detected and localized in tissue sections using RNA-fluorescence *in situ* hybridization (FISH) and was carried out in study II. For localization of DDX4 transcript, RNAscope® technology was performed on PFA-fixed ovarian cortex from GRPs. Using the RNAscope® Multiplex Fluorescent Detection kit, signal of DDX4 transcript was detected and

further amplified by the help of TSA amplification. As positive control, probes targeting Ubiquitin C were included and as negative control, the absence of probe signal from bacterial gene DabB was confirmed. Nuclei were visualized with Hoechst and images were analyzed using confocal microscopy and ImageJ.

3.2.5 Steroid analysis

For the quantification of steroids produced in ovarian tissue cultures in study I, ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was performed as described earlier (Hao et al., 2018) using culture media samples from tissue previously cultured for seven days (Asadi Azarbaijani et al., 2015) and stored on - 80 °C. Media samples of day 4 and day 7 were quantified for the following steroids: cortisol, corticosterone, estradiol, androstenedione, 17a-hydroxyprogesterone, progesterone, dehydroepiandrosterone, dihydrotestosterone, and testosterone. As negative controls, blank media samples were included.

3.3 OVARIAN CORTEX SINGLE CELLS

3.3.1 Tissue dissociation into single cells

Prior to all single-cell experiments in study II, frozen/thawed ovarian cortex was dissociated into single-cell suspensions. This method was established using bovine ovarian cortex which allowed for extensive testing of various digestion enzymes, enzyme mixtures and incubation times.

For dissociation of adult human ovarian cortex into single cells, tissue was minced into small pieces using scalpels and enzymatically digested using 1 mg/mL Collagenase I A, 50 µg/mL Liberase^{TM} and 100 U DNase I in a shaking water bath for a maximum of 50 min at 37 °C. After blocking of enzymes using 10% FBS, cells were pelleted, resuspended and filtered through a 40 µm cell strainer. Cell number was determined using Moxi automated cell counter with a size setting of 7.5 – 26 µm. Cell viability was subsequently analyzed using flow cytometry and DAPI to discriminate dead cells.

3.3.2 Fluorescence-activated cell sorting and flow cytometry

Fluorescence-activated cell sorting (FACS) and flow cytometry (FC) were performed for determination of cell viability, surface protein detection in single-cell suspensions and/or isolation of putative OSCs from frozen/thawed ovarian cortex in study II.

For FACS of putative OSCs, the previously published protocol was followed (Woods et al., 2013). Briefly, the single-cell suspension was serum blocked, followed by staining with DDX4 Ab (ab13840, Abcam) and subsequently with fluorescently labeled secondary Ab. All incubation steps were carried out for 20 min at 4 °C. The optimal DDX4 Ab concentration of 10 µg/mL was determined by titration experiments prior to and used for all subsequent DDX4 Ab staining. DAPI labeling was used for dead cell discrimination. Prior to FACS and FC, samples were filtered through FACS tube mesh of a size of 35 µm. DDX4 Ab-positive and Ab-negative cells were sorted based on fluorescence intensity using FACSAria Fusion.

For FC, the same staining protocol was followed with various different Abs targeting surface proteins. In the case of surface marker screening, the BD Lyoplate™ provided a panel of 242 surface markers and respective isotype controls in a 96-well plate format containing one marker per well. Where appropriate, a co-stain for DDX4 Ab was carried out. Surface marker expression was analyzed using CytoFlex S flow cytometer.

As negative controls in all flow experiments, secondary Ab only, fluorescence minus one (where appropriate) and isotype samples were included and used for analysis and/or sorting of populations of interest.

3.3.3 Immunocytochemistry

Protein expression in ovarian cortex single cells was analyzed by immunocytochemistry in study II. For this, cells were attached to glass slides using Cytospin before being fixed in PFA for 10 min on room temperature. Once the samples have dried fully, cells were permeabilized with Triton X and serum blocked. Primary Abs diluted in blocking solution were applied to glass slides at 4 °C over night. After thorough washing, slides were incubated with fluorescently labeled secondary Abs diluted in blocking solution. Hoechst was used as nuclear counterstain. After mounting, cells were imaged using confocal microscopy and analyzed in ImageJ.

3.3.4 Single-cell mRNA sequencing

Single-cell mRNA sequencing (scRNA-seq) provides information on the gene expression profiles of single cells at a given time point and was part of study II. Here, the 10x chromium platform was used which enables sequencing of a large number of cells to relatively low costs when compared to other scRNA-seq methods. ScRNA-seq was performed on ovarian cortex from one unsorted GRP, three pooled unsorted C-sec patients as well as sorted DDX4 Ab-positive and Ab-negative cells from the ovarian cortex of three pooled GRPs. Viable cells were either enriched using the Dead cell removal kit (for unsorted cell samples) and magnetic beads

or using DAPI in FACS to discriminate dead cells (for sorted cell samples). Sorted and unsorted cell samples were prepared for GEM formation on the same day, respectively.
Prior to loading of cells into the chromium controller, an aliquot of single-cell suspensions was stained with Trypan Blue, cells were counted in a Bürker Chamber and viability ratio was determined to be taken into account for GEM formation. All libraries were sequenced on a NovaSeq 6000 using a S1 flow cell and one lane per sample. Sequencing reads were aligned to the human genome hg19 using STAR aligner (Dobin et al., 2013). After initial filtering using Cell Ranger, around 6,000 cells per sample were kept with a sequencing depth of around 70,000 reads per cell and further analyzed using Seurat in R for downstream analysis (Butler et al., 2018; Stuart et al., 2019). Only genes detected in at least three cells were considered.
Briefly, further data filtration was performed to remove potential doublets and cells of low quality by excluding cells with a mitochondrial gene expression of more than 25% and less than 200 or more than 7000 genes expressed. In total, around 24,000 cells were kept for gene expression and cluster analysis.

3.4 STATISTICS

In study I, statistical analysis was performed using SigmaStat package SPSS and R. Mann-Whitney U test was used for comparison of exposed and unexposed patients in the following experiments: follicle densities, follicle and oocyte nucleus diameters, steroid concentrations, fibrotic tissue areas, TUNEL signal and γH2AX foci numbers between the two groups. To compare the proportions of different classes between the groups, Chi-square test was used. Spearman's rank correlation was used to determine correlations between morphological measures and the following variables: age, cyclophosphamide equivalent does (CED), isotoxic dose equivalent (DIE) and steroid concentrations. In cases that more than one variable correlated with morphological measures, the major predictor was determined using forward stepwise regression analysis. Results are shown as median with interquartile range and considered statistically significant at p-values < 0.05.

For scRNA-seq analysis in study II, statistical analysis was performed using R. Marker genes discriminating the different clusters were selected among highly expressed genes (p-value $<$ 0.01 and log(fold-change) > 0.25) using Wilcoxon RankSum test.

4 RESULTS AND DISCUSSION

4.1 IMPACT OF CHEMOTHERAPY ON HUMAN OVARIAN FOLLICLES AND STROMA

Cancer patients undergoing chemotherapy and/or radiotherapy are at risk of premature ovarian insufficiency. Ovarian tissue cryopreservation is an established fertility preservation method and offered to adult patients falling into the high-risk group of potential fertility loss, with the possibility of OTT upon the patient's recovery and desire for a child. In the case of pediatric patients, indication for fertility preservation is given when assigned to anti-cancer treatment considered of very-high risk. Usually, first-line chemotherapy is regarded as low risk and as such, patients are not referred to undergo ovarian tissue cryopreservation. However, patients treated with a regimen that classified as low/medium risk at first might fall into the high-risk group when assigned to second-line chemotherapy treatments. In these cases, the patients have been exposed to (multiple rounds of) chemotherapy prior to ovarian tissue cryopreservation. A limited number of studies investigated the impact of chemotherapy treatment on ovarian follicles prior to ovarian tissue cryopreservation in pediatric and young adult patients (Abir et al., 2016; Asadi Azarbaijani et al., 2015; Duncan et al., 2015; El Issaoui et al., 2016; Fabbri et al., 2012; Familiari et al., 1993; Marcello et al., 1990; McLaughlin et al., 2017).

In study I, the impact of first-line chemotherapy on quantity and quality of ovarian follicles as well as the quality of ovarian stroma was evaluated. For this, we investigated a patient cohort (age 1 – 24 years) exposed to first-line chemotherapy prior to ovarian tissue cryopreservation (treated) compared to patients that have not been exposed to chemotherapy treatment at the time of tissue cryopreservation (untreated) using various methods (Fig.8). Of note, following the guidelines of the Nordic Society of Pediatric Hematology and Oncology (NOPHO 2013), none of the treated patients in our cohort fell into the very high-risk group and hence, no indication for ovarian tissue cryopreservation would have been given. This issue, among others, was also addressed by Shapira et al. (Shapira et al., 2020) and clarified in our response letter (Pampanini et al., 2020).

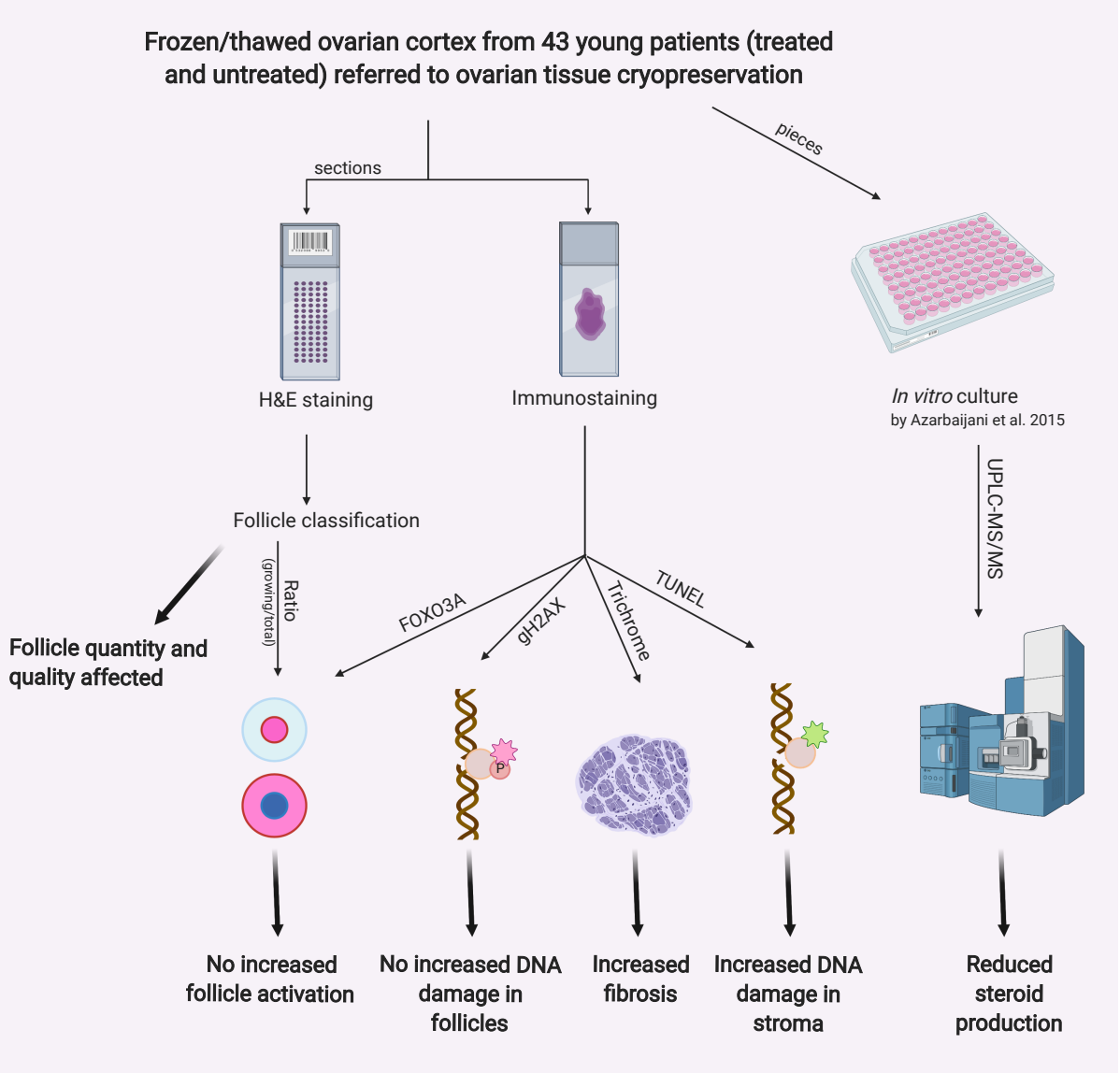

Fig.8. Study I design and main results. Frozen/thawed ovarian cortex from treated and untreated patients referred to ovarian tissue cryopreservation in Norway and Finland was either Paraffin-embedded and sectioned or cultured *in vitro*. To determine the impact of chemotherapy on ovarian follicles and stroma, sections were used for morphological analysis of ovarian follicles. Furthermore, immunostaining was performed to evaluate the extent of follicle activation, DNA damage and fibrotic lesions in ovarian tissue. To investigate steroid production in ovarian tissue, pieces of cortex were cultured. Culture medium of day 4 and 7 was analyzed in terms of steroid levels using mass spectrometry (UPLC MS/MS). In treated patients, follicles were found to be more atretic and smaller in size but neither follicle activation nor DNA damage was increased in follicles. Ovarian stroma of treated patients showed enlarged areas of fibrosis and DNA damage and steroid production was reduced compared to untreated patients. Graphics was created using ©BioRender.

4.1.1 Characterization of follicles

Morphometric analysis of ovarian tissue sections was performed to classify follicles based on health and developmental stage. While the total follicle density did not differ between treated and untreated patients, the density of atretic primordial and intermediary follicles was significantly increased and the density of intact primary follicles was significantly decreased in the treated patient group. The diameter of primordial follicles and their oocyte nuclei as well

as of intermediary follicles was significantly reduced compared to the untreated patients' follicles. Correlation analysis revealed a positive correlation of atretic (total, primordial and intermediary) follicles with CED and DIE, while age correlated positively with the density of atretic primordial follicles only. The density of intact primary follicles negatively correlated with CED and DIE. Furthermore, the size of follicle and oocyte nucleus negatively correlated with DIE.

In order to investigate whether primordial follicles of treated patients show an increased activation as suggested by Prof. Meirow's group's "burnout hypothesis", the ratio of intact growing follicles (intermediary and primary follicles) and total follicles (primordial, intermediary and primary follicles) was determined (Kalich-Philosoph et al., 2013). A significantly lower ratio of intact growing/total follicles was found in the treated patient group which is not in line with the expected scenario in the case of follicle activation. This reduction can be explained by an overall decrease of intact primary follicles observed in treated patients. To confirm this, FOXO3A as a marker of follicle activation was evaluated using immunohistochemistry: upon activation of follicles *via* PI3K/AKT signaling pathway, FOXO3A as downstream target translocates from the nucleus to the cytoplasm. Consistent with the previous finding, we found FOXO3A expressed in the nucleus in both groups, again indicating no chemotherapy-induced activation of follicles.

Furthermore, ovarian follicles of treated patients did not display an increased DNA damage. DSBs were studied by staining of phosphorylated H2AX (γH2AX) expression in oocyte nuclei using immunohistochemistry. No difference in the amount of γH2AX signal was found between treated and untreated patients.

4.1.2 Steroid production by ovarian tissue

Ovaries are regulated by and produce steroids *in vivo*. To investigate the functionality of ovarian tissue from treated and untreated patients, steroid concentration was determined in culture media of ovarian tissue cultured for seven days (Asadi Azarbaijani et al., 2015) using UPLC-MS/MS. The concentration of measured steroids increased in both patient groups over time (day 4 *versus* day 7), a sign of active steroid production in culture. However, significantly lower levels of estradiol, androstenedione, testosterone and progesterone were detected in treated compared to untreated patients. Further analysis revealed a positive correlation of steroid levels and density of intact primordial and intermediary follicles.

4.1.3 Analysis of ovarian stroma

To evaluate the health of somatic ovarian tissue cells surrounding the follicles, ovarian stroma from uncultured tissue was further evaluated in terms of DNA damage and prevalence of

fibrosis. Fragmented DNA in somatic cells was detected using TUNEL staining and revealed a larger area of TUNEL positive signal in the treated patient group to various degrees. Two of seven analyzed treated patients displayed rather low TUNEL signal that was comparable to levels of untreated patients, while the remaining five showed a significantly greater positive area when compared to untreated patients. Similarly, fibrosis as visualized by trichrome stain assay to detect newly formed, collagen-rich areas revealed significantly greater intensity and an enlarged area in the group of treated patients as compared to untreated ones.

4.1.4 Discussion

Qualitative and quantitative analysis of follicles and stroma cells in ovarian tissue revealed a negative impact of first-line chemotherapy exposure on ovarian tissue prior to cryopreservation for fertility preservation.

As it is inherent to retrospective cohort studies, a balanced design of samples is difficult, especially with this sensitive patient group and its precious study material. In this study, patient diagnoses were highly variable and so were the treatment regimens within the treated patient group. There was a significant difference in age between the two groups with the treated group being significantly younger compared to the untreated group. Furthermore, the time to tissue cryopreservation upon chemotherapy treatment varied between 7 – 55 days. Despite various degrees of chemotherapy exposure as reflected in variable CED and DIE values, none of the patients treated with first-line chemotherapy fell into the very-high risk group and would not have been offered ovarian cryopreservation following the current guidelines (NOPHO 2013). However, any drug-specific effect could not be studied here.

Morphometric analyses showed an increased density of atretic primordial and intermediary follicles upon chemotherapy exposure. In addition, intact primordial and intermediary follicles and the respective oocyte nuclei of primordial follicles were smaller in size in the treated patient group compared to untreated patients in our study and compared to follicle and oocyte nucleus sizes of the same age group in Westergaard et al. (Westergaard et al., 2007). Along with the observed decreased density of intact primary follicles in treated patients, a smaller size might reflect an impaired developmental potential of early follicles that survive chemotherapy treatment. This supports earlier findings of a lower *in vitro* developmental capacity of follicles from cultured ovarian tissue exposed to first-line chemotherapy before tissue cryopreservation (Asadi Azarbaijani et al., 2015).

To further investigate potential underlying mechanisms of the observed increased follicle atresia and decreased developmental potential, premature ovarian follicle activation and follicle apoptosis were evaluated.

Firstly, when analyzing the hypothesis of chemotherapy-induced follicular burnout (Kalich-Philosoph et al., 2013) in our study material, we did not detect an increased follicle activation based on morphological analysis (intact growing *versus* total follicles). Nor on molecular basis, as FOXO3A expression was restricted to the oocyte nucleus of primordial follicles indicating a quiescent follicle state. It should be considered that FOXO3A is only one of the targets of PI3K/AKT signaling and ideally, more players of this pathway should be investigated to draw firm conclusions, such as AKT, TSC1 and TSC2. Additionally, ovary cryopreservation was carried out within a few weeks after last chemotherapy exposure (in the case of FOXO3A stained tissue samples, patients had undergone ovary cryopreservation after 14 – 35 days) which could have led to us missing the time point of follicle activation, as suggested by Shapira et al. (Shapira et al., 2020). Re-localization of FOXO3A to the nucleus over time cannot be excluded from our study. Indeed, it was shown in a mouse study that nuclear localization of FOXO3A can be restored when oocytes are cultured under *in vitro* conditions that maintain dormancy (Nagamatsu et al., 2019). In humans, there is no evidence in literature yet to support a potential return back into dormancy once the follicle has been activated.

Secondly, DNA damage as an inducer of follicle atresia was investigated by immunohistochemistry of γH2AX. No difference in numbers of foci positive for γH2AX in intact primordial follicles of treated and untreated patients was found. Importantly, H2AX phosphorylation upon DNA damage was reported to take place within hours in rodents (Gonfloni et al., 2009; Petrillo et al., 2011; Rossi et al., 2017). Here again, considering the tissue sampling time (in the case of γH2AX stained tissue samples, patients had undergone cryopreservation after 8 – 55 days after chemotherapy exposure), it is likely that we missed differences in γH2AX signal. If we assume that the kinetics of DNA damage repair are conserved between mouse, bovine and human ovarian follicles, follicles had the chance to either resolve DSBs by repair mechanisms or undergo atresia during this time. PI3K/AKT signaling is considered a major pathway in maintenance of dormancy or trigger of primordial follicle activation. This pathway was reported to be disrupted in mice upon *in vivo* treatment with cyclophosphamide, mediating premature follicle loss through massive activation and burnout of the ovarian reserve within one week (Kalich-Philosoph et al., 2013). A recent study on bovine primordial follicles has revealed that PI3K/AKT pathway activation *via* PTEN inhibition hampers DNA repair mechanisms in dormant follicles. Ultimately, this led to unresolved DNA damage and potentially follicle death. These effects were found after 24 hours of tissue exposure *in vitro* (Maidarti et al., 2019). This would suggest that we did not find any differences in DNA damage of primordial follicles between treated and untreated patients because damaged follicles have already undergone atresia at the time of analysis and were classified as atretic follicles.

It must be acknowledged that we are looking at snapshots of the ovary condition at the time point of tissue fixation and thus ovary dynamics are being missed. The valid concern of an

overestimate of atretic follicle numbers due to tissue fixation is being debated continuously in literature. This concern should be kept in mind in fields that heavily rely on histological analysis, such as ovary research. In an ideal research situation, to fully understand how increased follicle atresia is mediated upon chemotherapy exposure in human, one should study ovarian tissue exposed to chemotherapy within one week at several time points and include various molecular key players of follicle activation (AKT, TSC1 and TSC2, FOXO3A, RPS6), DNA damage and apoptosis (γH2AX, CASP3, BCL2, PUMA) as well as DNA repair (MRE11, ATM, RAD51). Obviously, ethical concerns interfere with this hypothetical study design.

It is widely accepted that theca and granulosa cells up until secondary follicle stage are responsible for steroid production in the ovary. However, we still detected steroids in tissue culture medium when morphological analysis of ovarian tissue revealed very few secondary follicles and no antral follicles. Hence, steroid levels detected in the media of cultured ovarian tissue were rather produced by pre-antral follicles (intermediary and primary) or ovarian stroma cells. Lower steroid levels detected could be an indication for the reduced developmental potential of growing follicles or for a damaged stroma. With respect to the first hypothesis, we do see a reduced developmental potential of ovarian follicles in treated patients, as outlined above. With respect to the latter, we indeed detected a higher prevalence of fibrosis and increased DNA damage in the ovarian stroma of treated patients. Fibrosis is the process of excess deposition of extracellular matrix components, such as collagen, in tissue, often upon repeated injury, chronic inflammation or radiation, and is known to impair tissue cell function (Wynn, 2008). Furthermore, increased stiffness of ovarian tissue, e.g. induced by fibrosis, could explain the impaired developmental potential of early follicles in treated patients. Upon activation, follicles migrate from the rigid microenvironment within the cortex towards the inner, softer region of the ovary, the medulla (Hsueh et al., 2015). More studies are needed to investigate whether an increase in fibrotic areas is also detected in the medulla of treated patients' ovaries as compared to untreated ones, like that seen in the cortex. It was shown recently, that collagen deposition in human ovarian cortex continuously increases and more strikingly, elastic fibre content decreases with age (Ouni et al., 2020), potentially contributing to the onset of menopause. Additionally, the extracellular matrix and its ability to compress surrounding structures was shown to provide environmental cues for maintenance of dormancy of mouse follicles *in vitro* (Nagamatsu et al., 2019). An increased collagen deposition due to fibrotic lesions could hence contribute to the development of premature ovarian insufficiency in chemotherapy-treated patients.

A fibrosis-induced impaired cell function along with greater numbers of apoptotic somatic cells found in this study might explain the observed reduced steroid production in ovaries of treated patients. This is in line with another study that showed reduced estrogen levels in culture medium of human ovarian tissue treated with chemotherapy agents as compared to untreated

ones (Oktem et al., 2007b). One should consider that any steroid levels measured in ours and other studies might only reflect the *in vitro* situation upon tissue culture. Presence of fibrosis and stromal apoptosis, however, was analyzed in uncultured tissue and is more likely to reflect the *in vivo* situation.

In summary, exposure to first-line chemotherapy prior to ovarian tissue cryopreservation significantly alters the quality of the tissue. These alterations are reflected in an impaired follicle morphology, steroid production and stroma quality. It is currently unclear whether this type of damage might be reparable *in vivo* upon OTT as at least in adults, no difference in terms of ovarian function recovery was observed between OTT with chemotherapy-exposed or -unexposed tissue (Poirot et al., 2019).

4.2 CHARACTERIZATION OF THE HUMAN OVARIAN CORTEX

In multiple centers worldwide, human ovarian cortex is cryopreserved for fertility preservation purposes, but its cell type composition had not been studied yet at the single-cell level. Several groups reported the existence of OSCs in the adult human ovarian cortex that have the potential to differentiate into oocytes (Clarkson et al., 2018; Ding et al., 2016b; Silvestris et al., 2018; White et al., 2012). These putative OSCs have been used for treatment of women struggling to conceive in specific clinics and could potentially provide revolutionary treatment options for cancer survivors, too.

In study II, we analyzed the ovarian cortex cells in general and the putative OSCs in particular by means of gene and protein expression analysis at the single-cell level (Fig.9).

4.2.1 Deconvolution of different cell populations

Using scRNA-seq, we found that the adult human ovarian cortex consists of six main cell types, namely oocytes, immune cells (including monocytes and T-cells), granulosa cells, endothelial cells, perivascular cells and stroma cells. Cortex cells from C-sec biopsies and GRP ovaries showed the same composition and a similar abundance of the identified cell types. Upon integration of scRNA-seq data from adult human ovarian medulla (Fan et al., 2019), we saw all cortical cell types also being present in the medulla, except for oocytes that were unique to the cortex data set. Additional cell types within the endothelial cell cluster, such as lymphatic cells, the immune cell cluster, e.g. B-cells, and theca cells observed in the medulla (Fan et al., 2019) were not found in the cortex. Furthermore, cortex granulosa cells did not cluster together with the different granulosa cell clusters in the medulla.

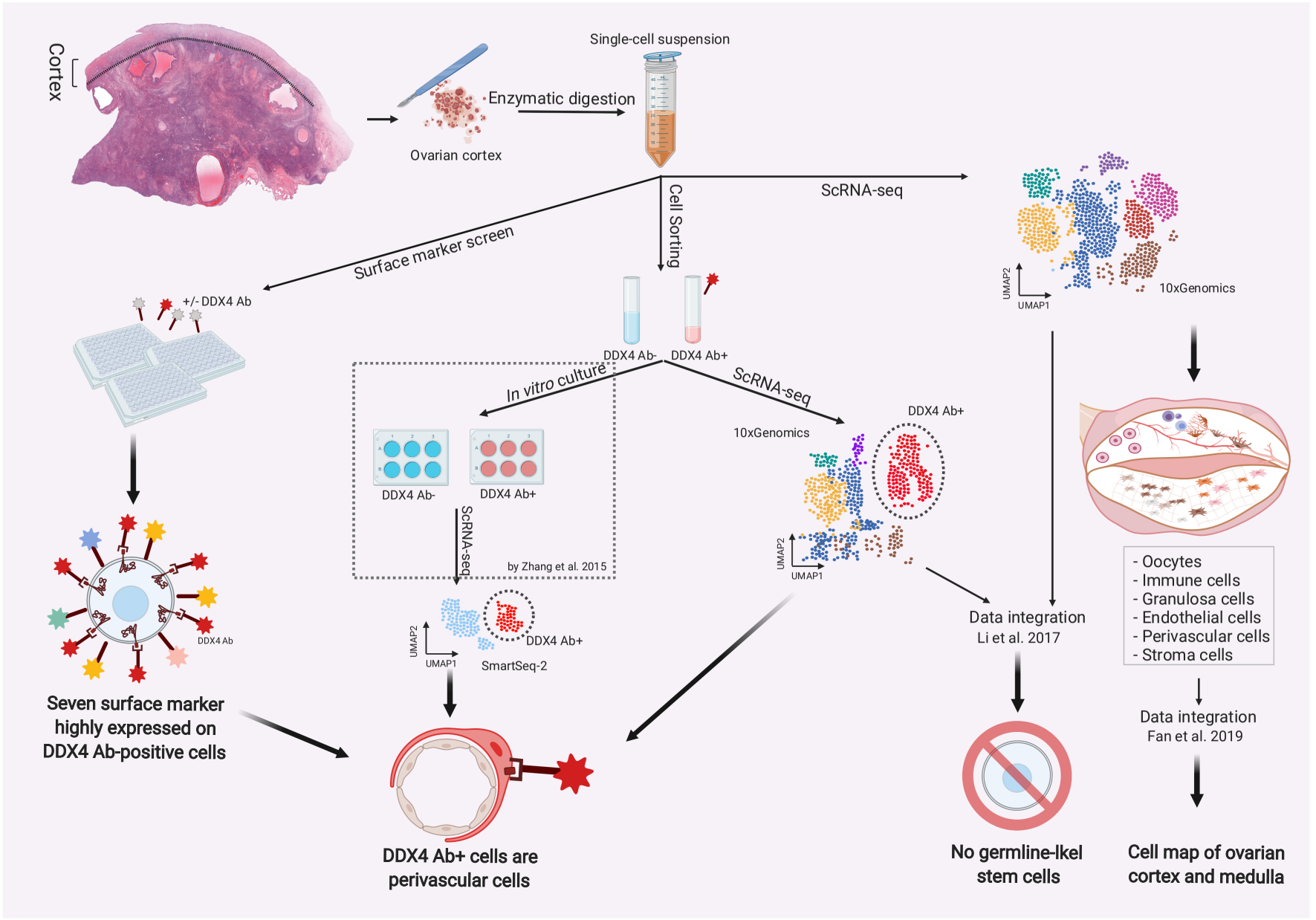
Cortex
Ovarian cortex
Enzymatic digestion
Single-cell suspension
Surface marker screen
Cell Sorting
ScRNA-seq
+/- DDX4 Ab
DDX4 Ab-
DDX4 Ab+
In vitro culture
ScRNA-seq
by Zhang et al. 2015
SmartSeq-2
10xGenomics
UMAP1
UMAP2
Seven surface marker highly expressed on DDX4 Ab-positive cells
DDX4 Ab+ cells are perivascular cells
Data integration
Li et al. 2017
No germline-lkel stem cells
- Oocytes
- Immune cells
- Granulosa cells
- Endothelial cells
- Perivascular cells
- Stroma cells
Data integration
Fan et al. 2019
Cell map of ovarian cortex and medulla

Fig.9. Study II design and main results. Ovarian cortex from GRPs and C-sec patients was digested into single cells using enzymes. Single ovarian cortex cells were either used unsorted for scRNA-seq analysis and surface marker screening or sorted into DDX4 Ab-positive and Ab-negative populations by FACS. DDX4 Ab-positive/negative populations were either scRNA-sequenced directly or cultured *in vitro* before subjected to scRNA-seq. Downstream analysis of scRNA-seq data revealed cell type information and integration of data sets (Fan et al., 2019; Li et al., 2017) was performed for further validation. Six main cell types could be distinguished among ovarian cortex cells. DDX4 Ab-positive cells were found to display characteristics of perivascular cells at the transcriptional and protein level. No cell with a gene expression profile similar to germline-like stem cells was found. Graphics was created using ©BioRender.

4.2.2 Characterization of DDX4 Ab-positive cells

We followed the previously reported protocol to isolate putative OSCs using FACS targeting DDX4 (Woods et al., 2013) and detected a small fraction of ovarian tissue cells (5 – 11.5%) to be positive for DDX4 Ab signal. Based on transcriptome profiling using scRNA-seq analysis, DDX4 Ab-positive cells revealed a perivascular cell nature with typical perivascular markers being highly expressed, such as *MCAM, TAGLN* and *CD9*, while *DDX4* was absent in these cells at the transcriptional level. At the protein level, DDX4 Ab-positive cells showed high expression of seven surface proteins as compared to the DDX4 Ab-negative fraction, including MCAM, CD44 and CD9, which could be mapped back to the perivascular cell cluster in scRNA-seq data. DDX4 Ab-positive and Ab-negative cells cultured under OSC conditions (Zhang et al., 2015) showed distinct gene expression profiles which overlapped with perivascular cells and stroma cells in the established single-cell map, respectively. *In situ* localization of *DDX4* mRNA by FISH further validated its expression in oocytes only, while perivascular cells did not express *DDX4.* Finally, immunohistochemistry on ovarian cortex sections and immunostaining of semi-digested ovarian cortex confirmed that DDX4 Ab signal is co-expressed with perivascular markers in cells of blood vessels. These results showed that DDX4 Ab used for isolation of putative OSCs in fact targets perivascular cells.

4.2.3 Search for putative OSCs

Unbiased and independently of DDX4 Ab-positivity, we sought to determine whether there are any cells residing in the adult human ovary that display a gene expression profile similar to putative OSCs or FGCs. ScRNA-seq technology is a powerful tool to detect small sub-populations and provides an unprecedented opportunity to dissect gene expression profiles of heterogeneous samples at single-cell resolution. While bulk mRNA sequencing easily overrides the gene expression profile from rare cells, scRNA-seq is sensitive for their detection. However, we acknowledged the fact that we could potentially miss out on cells showing an OSC- or FGC-like gene expression profile in our data set due to the expected low numbers of those cells (White et al., 2012). Hence, we integrated scRNA-seq data of female fetuses from a previously published fetal human data set (Li et al., 2017), thereby introducing – or increasing

the numbers of – cells with an FGC gene expression profile. Oocytes, granulosa cells and endothelial cells from the adult data set clustered with oogonia, late granulosa cells and endothelial cells from the fetal data set, respectively. However, no adult cells resembled female FGCs from the fetal data set in their gene expression profile. This integration analysis led us to conclude that there are no germline-like stem cells within the adult human ovarian cortex.

4.2.4 Discussion

Our single-cell map of adult human ovarian cortex cells in study II suggests that there are six main cell types. All of these cell types; oocytes, immune cells, granulosa cells, endothelial cells, perivascular cells and stroma cells; have been previously reported separately in various studies. These six cell types could be clearly distinguished from each other based on their transcriptional profile and were also mostly found in the inner, medullary region of the adult human ovary (Fan et al., 2019). However, previous reports on the existence of putative OSCs could not be confirmed here. Whether with the help of DDX4 Ab used for OSC enrichment (Woods et al., 2013) or following an unbiased bioinformatics approach by integrating female FGC transcriptome data (Li et al., 2017), none of the ovarian cortex cells showed a gene expression profile consistent with the transcriptome of OSCs or FGCs.

The single-cell map of ovarian cortex cells established in this study can serve as a reference for future research to study i.e. effects of aging, disease, chemical exposures, and treatments. Ideally, ovarian tissue from healthy, untreated women of reproductive age is being used to generate such a reference map. However, this type of tissue is hardly accessible and rarely available. Hence, cortex obtained from C-sec biopsies and GRP ovaries represent good alternatives and are widely used in human ovary research. For example, ovarian tissue from GRPs has also been used when human OSCs were originally reported (White et al., 2012). An important aspect to consider is that both donor types are under a special endocrine environment at the time of sampling due to either pregnancy or androgen treatment, respectively. It was shown that follicles in GRP ovaries do not differ in number, distribution and quality when compared to untreated women (De Roo et al., 2017), and pregnancy can still be achieved upon discontinuation of hormone treatment (Light et al., 2014). Hence, upon androgen treatment, the endocrine and reproductive potential of ovaries from GRPs is not fully lost but temporarily suppressed. In our study, we found the same cell type composition and a similar cell type abundance in ovarian cortex from C-sec patients and GRPs, indicating that the driving transcriptome profile of each cell type was not affected by treatment. Importantly, the same cell types were identified in the human ovarian medulla from cancer patients that have not undergone any treatment prior to ovary sampling (Fan et al., 2019). However, more in-depth analysis is needed to investigate if the abundance of certain cell types might be affected and if so which signaling pathways might be altered upon the respective hormone treatment, especially in steroidogenic cell types such as oocytes and granulosa cells.

The fact that we found cortical granulosa cells clustering separately from the medulla upon integration of the medulla data set by Fan and colleagues probably represents the different parts of ovarian tissue used in the respective studies. Granulosa cells in primordial follicles (more likely to be found in our cortex data set) are known to express a different gene expression profile compared to granulosa cells in growing follicles (more likely to be found in the medulla data set) (Ernst et al., 2018). Indeed, the granulosa cells in the cortex clustered together with fetal late granulosa cells confirming their identity as primordial follicle granulosa cells.

DDX4 Ab as a tool for OSC isolation has been debated for many years now and so far, no *in silico* studies can confirm that an external epitope is to be expected in the human DDX4 protein (Zarate-Garcia et al., 2016). However, experimental studies by us and others show that DDX4 Ab indeed binds to a fraction of unpermeabilized ovarian tissue cells in flow analyses. When HEK932T cells were transfected with a construct expressing the C-terminal (potentially external) end of DDX4, DDX4 signal was detected in the cell nucleus, the cytoplasm and indeed also on the cell surface (Clarkson et al., 2019). In our study, cells isolated following the OSC isolation protocol (Woods et al., 2013) showed a gene and protein expression profile of perivascular cells. Perivascular cells include smooth muscle cells and pericytes that were reported to originate from the same stem cell progenitor (Kumar et al., 2017). In the endometrium for example, MCAM-positive pericytes have been suspected to serve as a tissue progenitor cell population required for endometrial regeneration (Spitzer et al., 2012). In the fetal mouse testis, multipotent perivascular progenitor cells were observed to give rise to smooth muscle cells, pericytes and Leydig cells, the male counterpart of theca cells (Kumar et al., 2018). Depending on their tissue of origin, perivascular cells display proliferative and regenerative characteristics which had been exploited in several *in vitro* differentiation assays (Avolio et al., 2017) and in regenerative therapy (Cathery et al., 2018). This could explain the observed proliferative capacity of DDX4 Ab-positive cells upon culture under OSC conditions *in vitro* (Ding et al., 2016b; Silvestris et al., 2018; White et al., 2012). OSC culture conditions vary slightly between the different studies but usually include an irradiated embryonic feeder layer and multiple growth factors to provoke proliferation and prevent cell differentiation, such as leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Whether perivascular cells isolated from the adult ovarian cortex could potentially be differentiated into oocyte-like cells using above mentioned culture conditions remains to be investigated.

In order to investigate the existence of OSCs in the adult human ovary independently of DDX4 Ab, we performed pure bioinformatics analysis. For this, the previously published single-cell data set of human female FGCs (Li et al., 2017) was combined with our adult cortex datasets. Single-cell transcriptomic technologies are booming and provide powerful tools to deconvolute heterogeneous tissues at the single-cell level, especially when investigating small subpopulations. The integration of data sets across different platforms is a potent way to

validate and strengthen findings and is, with more and more single-cell data sets being available, more widely applied (Adey, 2019). However, this increased sensitivity of scRNA-seq that provides us with such great detail on rare events comes with its limitations. One of it is the requirement for tissue digestion into single-cell suspension prior to scRNA-seq. Usually, tissue digestion is performed by a combination of mechanical and enzymatic disruption. In fact, this applies to all single-cell analyses, including scRNA-seq and FACS. Protein expression is considered more robust if certain enzymes are avoided, e.g. Trypsin which has a high proteolytic activity potentially leading to false negative results in flow analysis. However, it was reported earlier that prolonged handling times and enzyme treatment can affect the transcriptional profile of cells (van den Brink et al., 2017). Furthermore, different tissue digestion protocols might enrich for certain cell types. Optimally, the digestion protocol yields a viable single-cell suspension that represents all cell types present in the tissue without altering their gene expression profile nor their abundance relative to *in vivo*. In reality, this is difficult to assess and control for and careful optimization of handling protocols has to be ensured. Subsequent enrichment of cells by different sorting strategies, such as FACS, has been shown to be another potential stress factor to the cells and might induce altered gene expression profiles (van den Brink et al., 2017). Here, we established a tissue dissociation protocol that yielded an adequate cell number and quality fulfilling the requirements for scRNA-seq using the 10x platform (e.g. > 80% viable cells). In fact, the cell type composition of ovarian cortex was very similar to the human ovarian medulla (Fan et al., 2019) and the same as recently reported in a single-cell study on non-human primate ovaries (Wang et al., 2020). All three studies used a different approach of tissue digestion into single cells.

In our study, we successfully and consistently identified expected somatic cell types as well as rare events, such as oocytes. However, certain cell types, such as neurons and epithelial cells have been previously reported to be found in the ovary but could not be detected in our data set. Reports about the presence of neurons in the human ovary are rare. Their presence was shown by immunohistochemical staining of tissue sections and were localized to the medullary region (Anesetti et al., 2001). In the mouse, neurons are mainly found in the medulla, too, with projections into the cortex (McKey et al., 2019). Intact neurons with their long cellular projections are difficult to isolate by standard tissue digestion protocols and are preferably analyzed by single-nuclei mRNA sequencing (Nguyen et al., 2018). The to date uncertain presence of neurons in the human cortex together with their unique cell morphology that makes them vulnerable to tissue digestion might explain the absence of neurons in our data set. In addition, epithelial cells known to cover the ovarian cortex and making up the ovarian surface epithelium (Auersperg et al., 2001) were missing in our data set. This one external layer of single cells is fragile, and we see it easily coming off when handling the ovaries. After ovary trimming, freezing, thawing and dissociation of the cortex, we might have lost those few epithelial cells expected to be found on a 10x10 mm piece of ovarian cortex. These missing cell types, however, do pose the question if other cell types got lost during sample preparation

and whether the observed absence of cells displaying a transcription profile similar to FGCs is real or due to technical limitations. Recently, critique was raised with regards to methodologies used in our sample preparation (Bhartiya et al., 2020). The authors claim that OSCs could not have been detected due to a low centrifugation speed. However, it needs to be highlighted here that Bhartiya and colleagues report stem cell populations among the mammalian ovarian surface epithelium that are isolated using a centrifugation speed of 1,000 *g* (Parte et al., 2011; Patel et al., 2018). These populations differ in location (ovarian surface epithelium *versus* ovarian cortex) and characteristics compared to the putative OSCs identified by Prof. Tilly and other groups (Clarkson et al., 2018; Silvestris et al., 2018; White et al., 2012). In order to be coherent with the previously reported studies on putative OSCs from adult human ovarian cortex, we followed the details regarding the centrifugation speed recommended in the original protocol (Woods et al., 2013).

5. CONCLUSIONS

In this thesis, we studied the impact of chemotherapy on pediatric and young adult human ovaries of cancer patients. Quality and quantity of ovarian follicles and quality of ovarian stroma were analyzed with regards to chemotherapy treatment prior to ovarian cryopreservation and compared to untreated cancer patients' ovarian tissue. Furthermore, we assessed the cell type composition of the adult human ovarian cortex with a particular focus on previously reported OSCs, isolated using FACS targeting DDX4. We characterized these DDX4 Ab-positive cells and investigated a possible existence of germline-like cells in the adult human ovary.

The main findings of these studies are listed below.

- Chemotherapy exposure alters quantity and quality of follicles in the ovarian tissue of pediatric and young adult ovaries: exposure to chemotherapy prior to tissue cryopreservation increases the density of atretic primordial and intermediary follicles and decreases the size of intact primordial and intermediary follicles and their oocyte nuclei. It also decreases the density of intact primary follicles.

- Neither follicle activation nor DNA damage in follicles could be determined with certainty as the main underlying mechanism of observed ovarian follicle alterations following chemotherapy treatment.

- Chemotherapy exposure alters the ovarian stroma of pediatric and young adult ovaries. An increased DNA damage and large fibrotic areas were observed in patients exposed to chemotherapy prior to cryopreservation.

- Six main cell types were found to constitute the cortex of adult human ovaries from GRPs and C-sec patients: oocytes, immune cells, granulosa cells, endothelial cells, perivascular cells and stroma.

- All somatic cell types are present in both the cortex and the medullary region of the adult human ovary. However, a specific sub-population of granulosa cells was found in the cortex. In addition, oocytes were found in the cortex.

- Putative OSCs isolated from the adult human ovarian cortex using DDX4-targeted FACS do not express germline markers but display a perivascular cell nature.

- No cells resembling germline stem cells could be detected in the adult human ovarian cortex by single-cell transcriptome analysis.

Following our findings in study I, ovarian tissue cryopreservation should be performed prior to exposure of chemotherapy if the patient's condition allows to avoid the negative impact on ovarian follicle and stroma health.
Following our findings in study II, the current dogma of a limited female ovarian reserve still holds true and does not motivate the existence of germline-like stem cells in the adult human ovary *in vivo.*

With these studies, we make small steps towards a better understanding of the human ovary. However, many more steps are to come to fully unravel the complexity of this organ and the great potential it holds as a paramount piece in the puzzle called female fertility.

6. FUTURE PERSPECTIVES

Many see the ultimate solution to female fertility-related problems being the *in vitro* production of human oocytes. The development of a robust system to derive mature and functional oocytes (either from pluripotent stem cells or immature primordial follicles) would pave the way to novel fertility treatment options. Not only would it eliminate the potential risk of re-introducing malignant cells in OTT in cancer survivors but could also serve as a much-needed test system for molecular signaling pathways involved in folliculogenesis, and compounds suspected to be toxic to female germ cells, such as chemotherapy agents and endocrine disruptive chemicals.

In mouse, the possibility to generate offspring from *in vitro* derived (from PGCs, iPSCs and ESCs to primordial follicles) (Hikabe et al., 2016; Morohaku et al., 2016) and *in vitro* grown oocytes (from primordial to fertilizable oocytes) (Eppig et al., 1996) was successfully proven. In human, first reports of *in vitro* derived oocytes (from iPSCs to oogonia) (Yamashiro et al., 2018) and *in vitro* matured oocytes (from cortical follicles to Metaphase II oocytes) (McLaughlin et al., 2018) hold tremendous promise. Several research groups are actively working on the development of systems to mature follicles in the laboratory.

One strategy lies in the construction of artificial ovaries (Chiti et al., 2018; Laronda et al., 2017; Pors et al., 2019). Artificial ovarian constructs have been proposed as a scaffold providing an appropriate environment (matrix and somatic/stromal cells) for ovarian follicle survival and growth *in vitro*. It is known that for primordial follicles to mature, a migration from the stiff cortical area towards the looser medullary region is necessary (Nagamatsu et al., 2019). For sure, this architecture of varying rigidity will be crucial for successful folliculogenesis *in vitro*. The Woodruff lab has demonstrated first successful reimplantation of such constructs in mice resulting in ovulated and fertilizable oocytes with healthy offspring (Laronda et al., 2017). The ultimate proof of a fully matured, *in vitro* derived human oocyte will be to fertilize it and investigate embryo morphology and blastocyst development *in vitro* based on criteria used in Reproductive Medicine centers.

Despite the field moving forward, we still encounter big knowledge gaps in fundamental questions regarding ovary biology, such as:

- Why are humans born with an ovarian reserve comprised of millions of primordial follicles that starts to decline already before birth? Do atretic follicles provide any function prior to their death?

- Is there an inherent difference between follicles destined to undergo atresia and follicles that achieve maturity? Is there a difference between follicles in the pre-pubertal and the adult ovary or is the developmental potential of all follicles the same? If there is a

difference between pre-pubertal and adult ovarian follicles, is it inherent or determined by their surrounding niche that is changing with age?

- Are there OSCs in the adult ovary and if so, what is their physiological role? Why do women undergo menopause if there was a constant supply of new follicles from OSCs?

I believe that understanding the differences between pre-pubertal and adult ovaries is of utmost importance and will provide more insight into some basic questions raised above. For example, single-follicle RNA-sequencing of different stages (primordial, primary and secondary) isolated from pre-pubertal and adult ovaries could give us first hints regarding inherent properties and developmental potential that might change during reproductive aging. In a recent study, ovaries of non-human primates were investigated with regards to aging and discovered the role of antioxidant pathways in age-related fertility decline (Wang et al., 2020).

Additionally, comparing the diversity of ovarian cell types within the stromal compartment of pre-pubertal and adult ovaries might reveal some insights into underlying mechanisms involved in follicle atresia, follicle activation or menopause. Using our established single-cell map of adult ovarian cortex as well as the published single-cell map of adult ovarian medulla (Fan et al., 2019), scRNA-seq of pre-pubertal human ovary could be indicative of crucial differences in somatic cell type and ovary niche composition. We are currently observing how the stem cell niche is being discovered and its importance revealed in other human organs. The niche, or cellular environment, of tissue stem cells emits cues to either maintain their quiescent state or promote their differentiation. Similarly, in the ovary, it would be interesting to study the stromal cell type complexity. Do we see certain stromal cell types in proximity to primordial follicles maintaining their dormancy compared to those that have been activated and start growing?

Once we better understand the ovarian follicles' microenvironment in a healthy state at different ages, we might also be able to i) mimic those conditions *in vitro* and ii) protect the ovaries from damage e.g. induced by anti-cancer therapy, endocrine disruptors or aging.

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