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**Potential protective effect of microRNA 10a to prevent
chemotherapy – induced ovarian damage in mouse model**

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**«POTENTIAL PROTECTIVE EFFECT OF MicroRNA 10a TO
PREVENT CHEMOTHERAPY – INDUCED OVARIAN DAMAGE
IN MOUSE MODEL»**

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ABSTRACT

It is well-known that cancer and its treatments can negatively affect female fertility by causing ovarian damage and massive activation of the primordial follicles. Today, several methods are available to preserve fertility, like oocyte, embryo and ovarian tissue cryopreservation but these techniques cannot be applied to all of the patients. Hence, there is a need to develop new strategies and the pharmacological approach seems to be very attractive. Focusing on this direction, the use of miRNAs in female fertility preservation strategies appears to be quite innovative and promising.

This study is based on the concept that miRNAs change their expression profiles during exposure to chemotherapy hence the goal is to identify specific miRNAs with ovarian protective properties during oncological treatments. For this purpose miR-10a was examined as a potential target. In a previous study C. Alexandri showed that miR-10a was significantly down-regulated in the PND3 mouse ovaries after exposure to chemotherapy (4-HC) for 1-24h. Based on this observation we tried to restore its levels of expression into mouse ovaries *in vitro* in order to decrease chemotherapy-induced damage.

For this reason, 64 ovaries were isolated from PND3 mice and used for liposome-mediated miRNA-mimic transfection experiments. More specifically four groups were created: control (culture medium alone), chemo (1h/4-HC/20 μ M), miR-10a mimic (culture medium+miR-10a mimic) and chemo + miR-10a mimic (1h/4-HC/20 μ M + miR-10a mimic). The expression levels of miR-10a were quantified by qPCR to confirm the efficiency of the transfection. The levels of apoptosis in the ovary were assessed by TUNEL assay while the expression of genes related to apoptosis was evaluated by qPCR. The ovarian follicle development and growth were evaluated by histological studies while the levels of proliferation were assessed by Ki-67 staining and qPCR assays for genes related to cell proliferation.

The transfection with miR-10a was proven to be successful as the expression of miR-10a was increased compared to control whereas the levels of its targeted genes PTEN were decreased. However, there was no clear evidence that miR-10a up-regulation protects the ovary from apoptosis or affects directly the follicle activation. Nevertheless, more studies have to be conducted, including studies in *in vivo* mouse model, to elucidate the role of miR-10a in the ovary after chemotherapy exposure.

INTRODUCTION

The first evidence of cancer originates from Hippocrates (460-370 BC), a Greek physician who has defined the history of medicine. In his famous ‘Hippocratic Corpus’, he first established the term ‘karkínos’ (καρκίνος=crab) to describe a chronic disease characterized by a progressive uncontrolled expansion of some tissues that resembled the leg movement of crabs. The term was later adopted in Latin as ‘cancer’ and is still at use nowadays (Salaverry, 2013). Cancer represents a group of diseases which share a main common characteristic, the constant unregulated cell proliferation. Cancer cells have lost their ability to properly respond to their environmental signals, thus they divide uncontrollably (Cooper & Hausman, 2007).

Although cancer is a dangerous and often fatal condition, over the past decades there has been a remarkable progress in cancer patients’ care and treatment, leading to an encouraging increase in their prognosis. However, despite the therapeutic effect of the current oncological treatments, they also have numerous side effects. Chemotherapy is a widespread method in cancer treatment, which has potent cytotoxic effects and can also be harmful to the healthy cells, causing adverse consequences.

The ovaries are particularly susceptible to chemotherapeutic treatment resulting in negative effects varying from temporary amenorrhea to long term infertility and premature ovarian failure (Guzy & Demeestere, 2017). Hence, there is a need to overcome the challenge of chemotherapy-induced infertility and give to female cancer survivors the opportunity to experience the beauty of motherhood. Despite the fact that there are available methods to preserve fertility, they cannot be applied and have successful outcome in all of the patients. As a result, new, less invasive and more efficient fertility preservation strategies have to be developed.

Focusing our interest on the pharmacological protection of the ovaries during oncological treatments, the regulation of genes expression involved in pathways that can be triggered by chemotherapy seems to be quite attractive. The expression of genes involved in apoptosis, DNA damage response and follicle activation can be regulated by microRNAs. These small single stranded non-coding RNAs, play a vital biological role as they target more than 60% of human genes (Hogg & Harries, 2014). MicroRNAs act by down-regulating the expression of their target genes in a tissue-specific manner and this unique characteristic makes them interesting molecules in

developing new cancer therapeutic drugs but also innovative methods of fertility preservation.

CHAPTER 1 – MAMMALIAN OVARIAN FOLLICLES

1.1. The process of folliculogenesis

Follicles are the functional units of the ovary. In primates each follicle contains one oocyte surrounded by one or more layers of granulosa cells (GCs) depending on the maturation stage. Between the oocyte and the granulosa cells lays a membrane named basal lamina (Oktem & Oktay, 2008). There is constant communication between the oocyte and its surrounding somatic cells, served by gap junctions, and it allows the exchange of inorganic ions, second messengers, and small metabolites, which are essential for the survival and development of the oocyte (Kidder & Mhawji, 2002). Follicles can be categorized by their developmental stage, according to Gougeon's classification (Gougeon, 1996). Small quiescent follicles compose the stock of resting follicles, also referred to as ovarian reserve. The ovarian reserve consists of all oocytes potentially available for fertilization throughout the fertile lifespan. These follicles are destined to either ovulate or undergo apoptosis, in a process called atresia. This pool of resting follicles varies between different species and individuals of the same species. In human it is established before birth and continuously decreases with age progression until it is depleted (Findlay et al., 2015) (Fig. 1.1.). On the contrary, the ovarian reserve in mice is formed shortly after birth. From 12.5-13.5 DPC oogonia start to form nests (or clusters) via which they interact with somatic cells, proliferate and exchange mRNAs, microRNAs, proteins and organelles. The breakdown of oogonia nests into single oocytes starts before birth and it is completed by PND5 (Fig. 1.1.). During that time numerous oocytes die while the remaining ones form primordial follicles and constitute the mouse ovarian reserve (Monget et al., 2012; Findlay et al., 2015).

The maturation of the follicle proceeds from primordial to primary and then secondary stages of development until ovulation (Fig. 1.2). The growth phase of the primordial follicles begins with the recruitment of GCs to proliferate and the increase in the oocyte's size. Soon, a thick layer of glycoproteins called zona pellucida starts to surround the oocyte. A secondary follicle begins to shape and the ovarian stroma differentiates into two parts called theca externa and theca interna. At this stage the follicle is called preantral until the appearance of a small fluid-filled cavity of 40 µm

that aggregates to form the antrum. The GCs change in morphology from flattened to cuboidal appearance and form more layers around the oocyte. After that the secondary antral follicle continues to grow in size due to cell proliferation and fluid accumulation until ovulation (Gougeon, 1996). The corpus luteum is formed after ovulation from cells of the follicular wall. Corpus luteum is a small transient endocrine gland which produces mainly progesterone and degenerates after a brief period of time, if fertilization does not occur (Tomac, Cekinović, & Arapović, 2011).

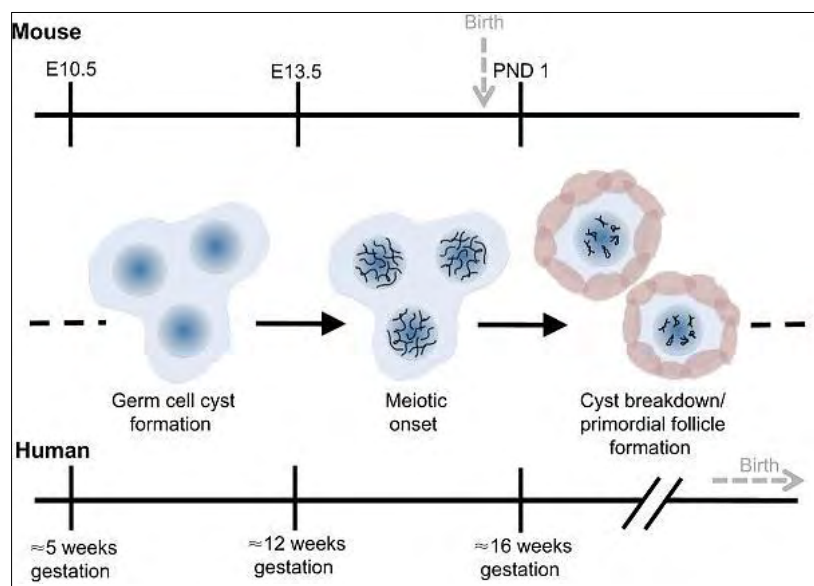


Figure 1.1. Comparative timelines of the events that lead to the ovarian reserve formation in mice and humans (Grive & Freiman, 2015).

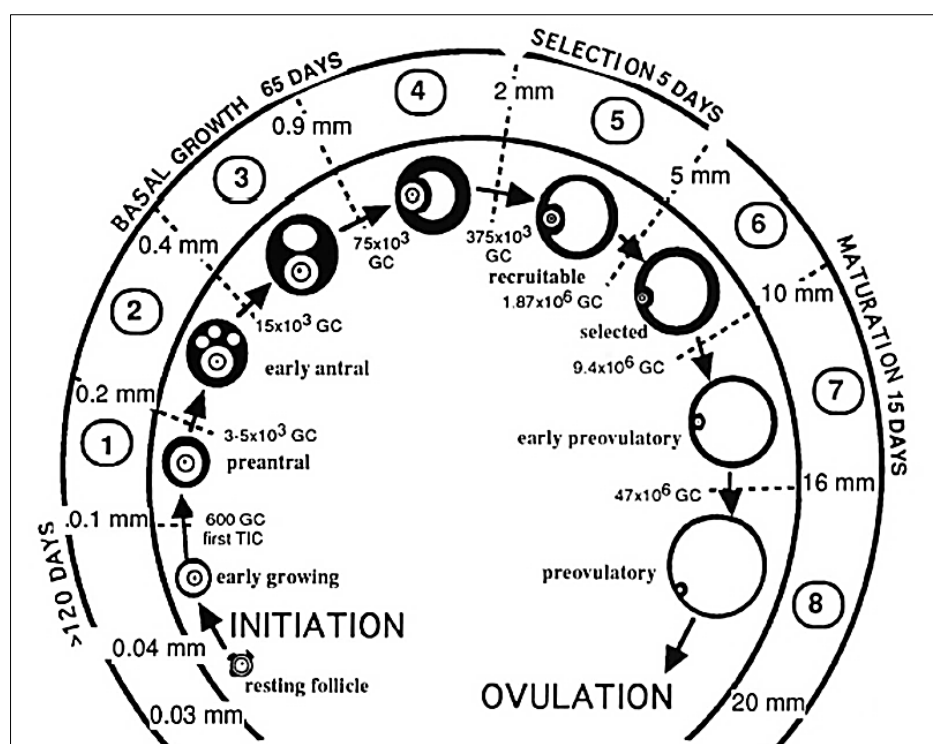


Figure 1.2. Stages of human follicular development according Gougeon's classification (Gougeon, 1996).

1.2. Follicle activation

Primordial follicle activation involves the proliferation and differentiation of PGCs as well as the growth of the oocyte (Liu K. et al., 2014). Previous studies have mentioned several signaling pathways that regulate the follicle activation. One of the most studied is the PI3K pathway, which is implicated in follicle activation as it enhances oocyte growth and stimulates the proliferation and differentiation of the surrounding GCs (McLaughlin & McIver, 2009) (Fig. 1.3.). PI3K signaling involves the phosphorylation of 4,5-biphosphate (PIP2) to 3,4,5-triphosphate (PIP3) by the active form of PI3K. PIP3 then recruits and activates Protein Kinase B (PKB or Akt), a protein that promotes the survival, growth and proliferation of the cells (Mabuchi et al, 2015). Several factors can regulate the activation and progression of this pathway in growing mouse oocytes (Table 1.1). Forkhead box O3 (FOXO3), a substrate of PI3K, has been reported to suppress follicle development, supposedly by maintaining

the dormant state of primordial follicles (Liu L. et al., 2007). This protein belongs to the class of transcription factors and it is present in the nucleus of quiescent follicles but it is exported upon activation allowing the progression of the maturation (McLaughlin & McIver, 2009). Another negative regulator of PI3K pathway is the phosphatase and tensin homolog (PTEN), which inhibits the pathway by dephosphorylating the second messenger PIP3. Oocyte-specific *Pten* deletion in mice led to massive activation and premature ovarian failure (POF) (Reddy et al., 2008). Similarly inositol polyphosphate-4-phosphatase (INPP4B) dephosphorylates PIP2, inhibiting the production of PIP3 and therefore blocking the progression of PI3K signaling independently from PTEN (Mabuchi et al., 2015). GCs actively participate in the regulation of the PI3K signaling cascade (Zhang & Liu, 2015). GC-secreted KIT ligand (KITL) acts on KIT receptors on the oocyte surface and triggers the PI3K signaling (Zhang & Liu, 2015).

The mTORC1 signaling pathway affects metabolism and cell growth by regulating functions such as protein synthesis, ribosome biogenesis and autophagy. In the ovary the mTORC1 cascade is stimulated by GCs of selected primordial follicles resulting in their proliferation and differentiation as well as the up-regulation of KITL (Zhang & Liu, 2015) (Fig. 1.3.). The mTORC1 signaling pathway is negatively regulated by an heterodimeric complex consisting of tuberous sclerosis complex 1 (TSC1) and tuberous sclerosis complex 2 (TSC2). Studies by D. Adhikari and his team associated *Tsc1* and *Tsc2* deletion with premature activation of all primordial follicles (Adhikari et al., 2010). These results reveal a correlation between mTORC1 signaling and the acceleration of follicle activation.

The p27^{Kip1} (p27)-cyclin dependent kinase (CDK) system is based on p27-mediated suppression of the cell cycle (Zhang & Liu, 2015). Normally p27 is specifically expressed in the nuclei of dormant oocytes whereas p27 deletion resulted in depletion of the total number of follicles, similarly to POF in humans (Rajareddy et al., 2007).

Nobox, *Sohlh1* and *Sohl2* exemplify the role of oocyte-specific genes in primordial follicle activation. NOBOX oogenesis homeobox (Nobox) is expressed in germ cell cysts and oocytes of primordial and growing follicles and is considered a decisive factor in activation control. Nobox deletion in embryonic stem cells resulted in disruption of early folliculogenesis in newborn mice (Rajkovic, Pangas, Ballow, Suzumori, & Matzuk, 2004). Spermatogenesis and oogenesis specific basic helix-loop-helix 1 (*Sohlh1*) is preferentially expressed in germ cell cysts, primordial and

primary follicles but cannot be detected in secondary follicles (Zhang & Liu, 2015). A study by S. Pangas and her colleagues concluded that the transient expression of SOHLH1 is crucial for oogenesis and oocyte development. The same study suggested that SOHLH1 regulates the expression of genes necessary for oocyte development, including Nobox (Pangas et al., 2006). A couple of years later a *Sohlh1*-null mouse model was created by the team of Choi. Similar phenotypes were described, including abnormalities in the molecular profile of primordial follicles and loss of oocytes early in post-natal life (Choi, Yuan, & Rajkovic, 2008).

Table 1.1. Major molecules implicated in the regulation of the PI3K pathway.

PI3K pathway activators	PI3K pathway inhibitors
mTOTO1	FOXO3
KIT – KITL	PTEN
	INPP4B

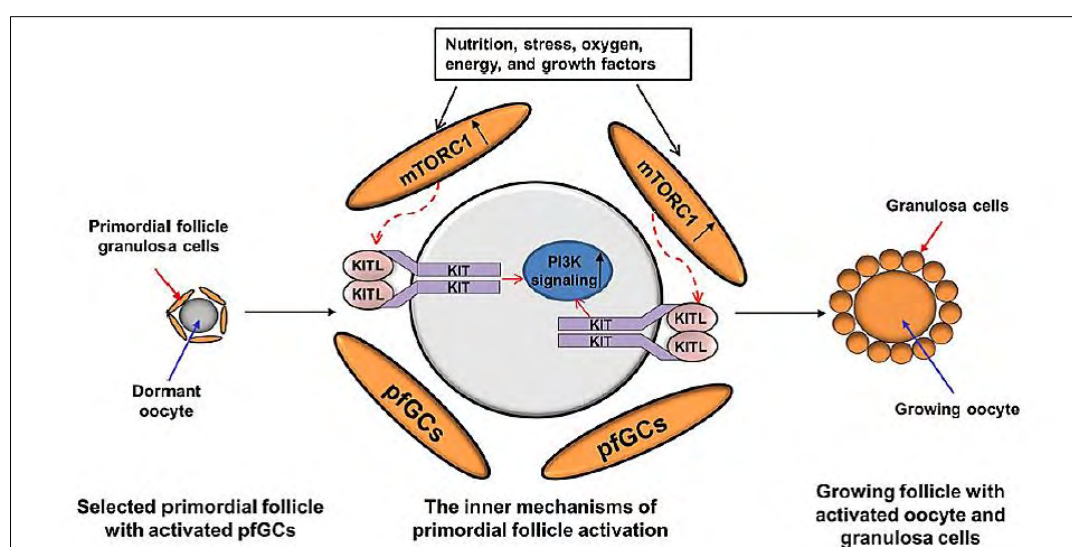


Figure 1.3. Current model of primordial follicle activation in the mammalian ovary. A variety of stimuli (nutrition, stress, oxygen, energy, growth factors) are responsible for the activation of the mTORC1 signaling pathway. mTORC1 activation in the GCs causes the up-regulation of KITL resulting in the stimulation of the PI3K signaling cascade. PI3K signaling awakens the dormant oocyte and triggers the proliferation and differentiation of the surrounding GCs (Zhang & Liu, 2015).

CHAPTER 2 – CHEMOTHERAPY TREATMENT & INFERTILITY

2.1. Definition of chemotherapy

Chemotherapy is a type of oncological treatment that uses drugs to stop the growth of cancer cells, either by killing them or by stopping their proliferation. There are multiple substances used as chemotherapeutic drugs. They are frequently categorized according to the cell cycle stage (cycle-specific agents) in which they are more active. Alkylating agents and platinum agents are categorized as cycle-non-specific because they are effective regardless of the cell cycle stage (Hoffman et al., 2012).

2.2. Cyclophosphamide

Alkylating agents are cycle-non-specific chemotherapeutic agents. Their action is independent of DNA synthesis in the targeted cells, and as a result they are effective from G0 to Mitosis. Their main mechanism of action is preventing cell division by intra- or inter- cross linking strands of DNA, leading to alterations in genomic structure and inhibition of its replication. These alterations can be single- or double-strand breaks that trigger the DNA damage response and lead to cell death when repairment is impossible (Chu & Rubin, 2012).

Cyclophosphamide is an alkylating agent widely used in cases of ovarian, breast and hematological cancers as well as in several autoimmune disorders (Jeelani et al., 2017). Cyclophosphamide is actually an inactive prodrug that requires enzymatic and chemical activation to become functional (Fig. 2.1.). The first step of the activation is catalyzed by P450 isoenzyme 2B (CYP2B) in the liver. The generated metabolites are 4-hydroperoxycyclophosphamide (4-HC) and its tautomer, aldophosphamide, which are then diffused into cells (Emadi et al., 2009). Aldophosphamide is further metabolized into phosphoramidate mustard, which is responsible for the cytotoxic effect of the drug and acrolein, which can cause severe off-target toxicity. Toxicity in several tissues has been attributed to cyclophosphamide exposure, including the bone marrow, bladder, gonads and even the heart in high doses (Emadi et al., 2009). Both

cyclophosphamide metabolites can be detoxified by glutathione (GSH) conjugation (Lopez & Luderer, 2004).

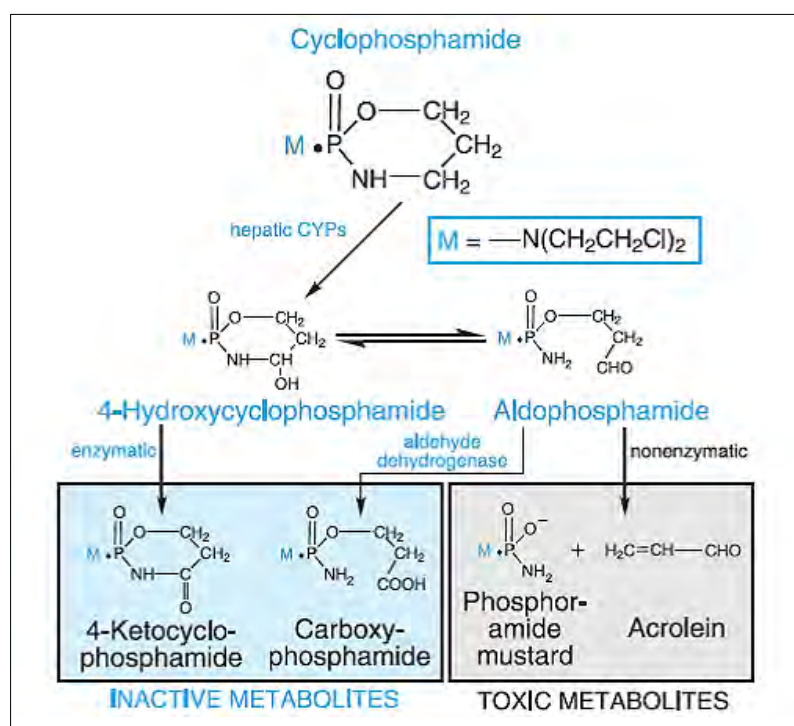


Figure 2.1. Metabolic pathway of cyclophosphamide (Chabner et al, 2006).

2.3. Chemotherapy – induced ovarian damage

Women are born with a defined number of follicles in their ovaries. Even though this theory was challenging, it has been established that the number is irreplaceable and declines with increasing age. However, it is well-known that cancer and its treatments, like chemotherapy, can affect the ovarian functions leading to compromised fertility. Cytotoxic therapies can cause from minor to severe gonadotoxicity depending on the drug used, the duration and dose of the treatment and the patient's age at the time of treatment (Ben-Aharon & Shalgi, 2012). Especially, alkylating agents like cyclophosphamide and its active metabolites are characterized as highly gonadotoxic and they have been proven to cause amenorrhea and infertility and they can lead to depletion of the ovarian reserve (Chu & Rubin, 2012) (Fig. 2.2.).

Even if the mechanisms of chemotherapy-induced ovarian damage are not yet fully understood, there are three possible explanations. The first scenario concerns the direct ovotoxicity that usually affects the granulosa cells (GCs) of growing and antral follicles, which are more susceptible as rapidly dividing cells (Ben-Aharon & Shalgi, 2012). This effect starts immediately at the onset of the treatment and typically causes temporary amenorrhea with short- or long-term consequences. The damage in growing follicles leads to a decrease in AMH levels; an hormone which normally suppresses the growth of the primordial follicles keeping them quiescent at the resting pool. The lack of this inhibitory signal can cause massive recruitment of the dormant follicles leading to premature ovarian reserve depletion. This is an indirect mechanism of chemotherapy induced ovarian damage which has been referred to as the ‘burn-out effect’ (Ben-Aharon & Shalgi, 2012; Morgan et al., 2012) (Fig. 2.3.). A third explanation can be the oxidative stress and glutathione depletion that have been observed as a result of chemotherapy exposure (Tsai-Turton et al., 2007; Jeelani et al., 2017). It has also been mentioned that chemotherapeutic drugs can induce changes in the capillaries and ovarian blood flow leading to vascular toxicity and to stroma and cortical tissue fibrosis (Ben-Aharon & Shalgi, 2012).

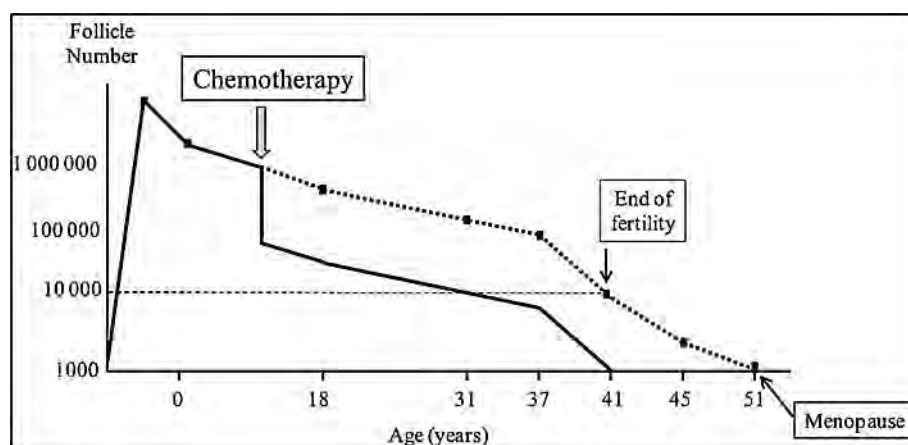


Figure 2.2. The physiological decline in the ovarian reserve compared to the decline after chemotherapy administration (Adapted from: Filippi et al., 2016).

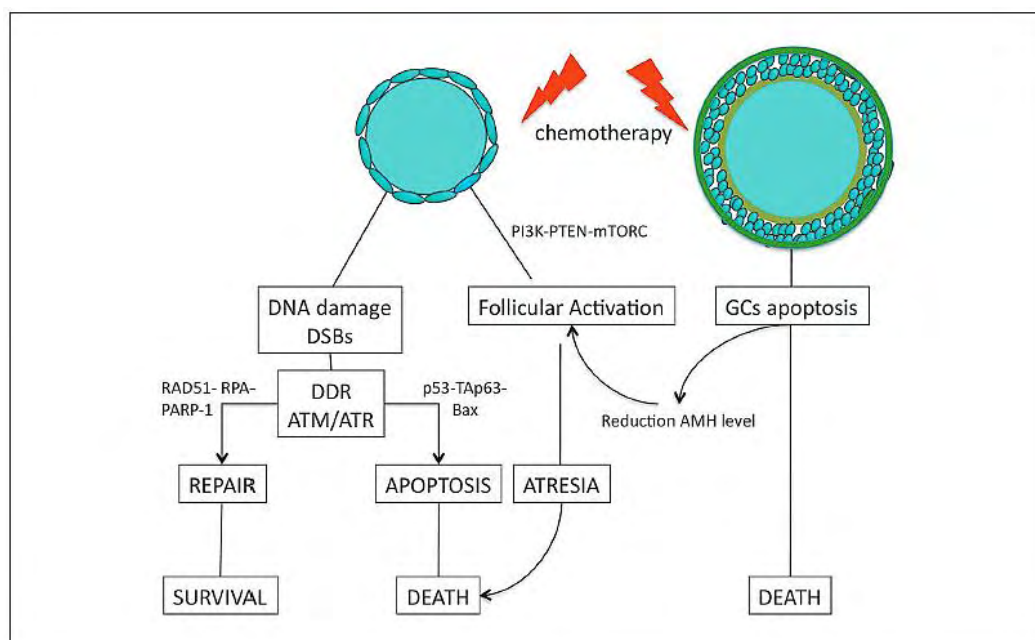


Figure 2.3. Mechanisms of chemotherapy-induced ovarian follicular depletion (Guzy & Demeestere, 2017).

CHAPTER 3 – APOPTOSIS

3.1. Apoptosis in the ovary

Apoptosis in the ovary is one of the most important and essential physiological processes (Hussein, 2005). Apoptosis occurs in ovarian cells from fetal life, when about 7×10^6 oocytes in the human ovary begin to decline progressively. According to Kerr et al., the average number of primordial follicles in the human ovary at ages 6-9 is 500000 and is reduced to 8000 by 44 years (Kerr, Myers, & Anderson, 2013). Considering that only one follicle ovulates in every menstrual cycle, the maximum number of follicles reaching ovulation in the human reproductive lifespan is ~450. Therefore it is obvious that a large number of follicles (~250000) are destined for apoptosis (i.e. atresia). Usually ~1000 follicles enter their growing phase in each menstrual cycle, however only 1 (rarely 2) makes it as the dominant follicle and continues growing while the rest are lead to atresia. After ovulation, apoptosis is also responsible for the regression of the corpus luteum, in a process named luteolysis (Hussein, 2005).

3.2. Mechanisms of apoptosis

Apoptosis can be defined as a biologically programmed and well-orchestrated cell death characterized by membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation. Eventually, the cell is reduced to small membrane-surrounded fragments (i. e. apoptotic bodies), which are phagocytosed by neighboring cells (Reed, 2000; Renehan, Booth, & Potten, 2001).

A variety of intracellular and extracellular stimuli can trigger apoptosis. Apoptosis occurs physiologically during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. Moreover, the immune system activates apoptotic pathways to remove damaged and infected cells. However, in pathological situations apoptosis occurs when the cells experience stress or DNA damage due to cytotoxic drugs and radiation (Elmore, 2007)..

There are two major apoptotic signaling pathways (Saraste & Pulkki, 2000; Su, Yang, Xu, Chen, & Yu, 2015): the intrinsic and the extrinsic. Both pathways activate caspase cascades, via initiator caspases 9 and 8 respectively. The intrinsic (mitochondrial) pathway is triggered by several intracellular stimuli such as DNA damage, radiation, cytotoxic drugs, p53 signaling, growth factor deprivation and oxidative stress. The main characteristic of this pathway is the synthesis of the apoptosome from procaspase-9, apoptotic protease activating factor 1 (Apaf-1) and cytochrome c. Numerous Bcl-2 family members (Bax, Bak, Bcl-2, Bcl-xL, Mcl-1, Bid, and Bim) are implicated in the pathway, acting as regulators of the mitochondrial membrane permeabilization (Fig. 3.1.). Pores formed on the outer mitochondrial membrane allow the leak of pro-apoptotic factors and cytochrome c out of the mitochondria, consequently activating procaspase-9.

On the other hand, the extrinsic (death receptor) pathway is directly related to the binding of death ligands (FasL, TRAIL, TNF- α and TWEAK) to death receptors of the TNF receptor superfamily (Fas, TNFR DR3, DR4 and DR5). In the case of FAS-FASL interactions, the death-inducing signaling complex (DISC) is formed, which is composed of FADD and procaspase 8. DISC then may act in one of the two ways: it either directly provokes cell collapse or cleaves Bid form tBid thereby triggering the intrinsic apoptotic pathway (Fig. 3.1.).

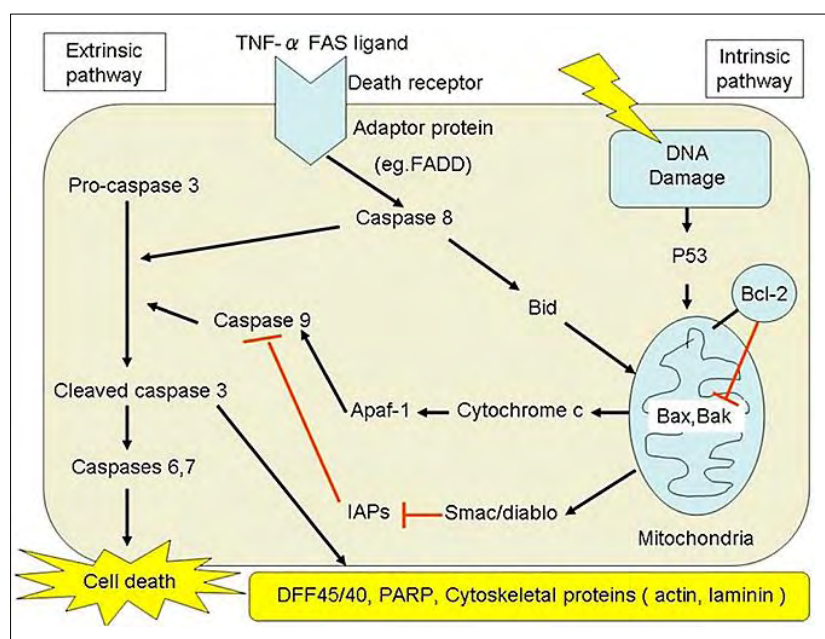


Figure 3.1. Diagram of the main molecules implicated in the extrinsic and the intrinsic apoptotic pathways (Loane & Faden, 2010).

CHAPTER 4 – MicroRNAs

4.1. MicroRNA biogenesis

MicroRNAs are single-stranded RNA molecules that usually consist of 22 nucleotides in their mature form and can be detected in all animal and plant organisms (Finnegan & Pasquinelli, 2013). The biogenesis process begins with the transcription of the MicroRNA gene. The genomic location of miRNAs is not specific. MiRNA genes may have their own promoter or they can be found in introns or exons of protein-coding or non-coding genes (Zhao & Srivastava, 2007; Finnegan & Pasquinelli, 2013). RNA polymerase II transcribes miRNA genes into primary miRNAs (pri-miRNA). The pri-miRNA transcript is afterwards cleaved by a Microprocessor complex composed of Drosha and DiGeorge Syndrome Critical Region 8 (DGCR8). This reaction results in the formation of a hairpin-shaped precursor miRNA (pre-miRNA) which is then transported out of the nucleus by a protein called Exportin 5. While in the cytoplasm the pre-miRNA hairpin is further processed by the Dicer complex; the loop is cleaved and one of the strands is removed and led to degradation whereas the remaining one is loaded onto Argonaute to form the miRNA-Induced Silencing Complex (miRISC) (Finnegan & Pasquinelli, 2013) (Fig. 4.1.).

The strand that participates in the miRISC formation is considered the mature miRNA sequence and it defines its targeted genes. A MicroRNA can target mRNAs that are either completely or most frequently partially complementary to its sequence (Finnegan & Pasquinelli, 2013). The interaction between the miRNA and the mRNA results in down-regulation of the gene expression either by translational repression or mRNA destabilization with subsequent degradation of the molecule (Zhao & Srivastava, 2007; Finnegan & Pasquinelli, 2013) (Fig. 4.1.). Some studies mention a third mechanism of action that includes direct target-transcript endonucleolytic cleavage catalyzed by the miRISC (Valencia-Sanchez, Liu, Hannon, & Parker, 2006).

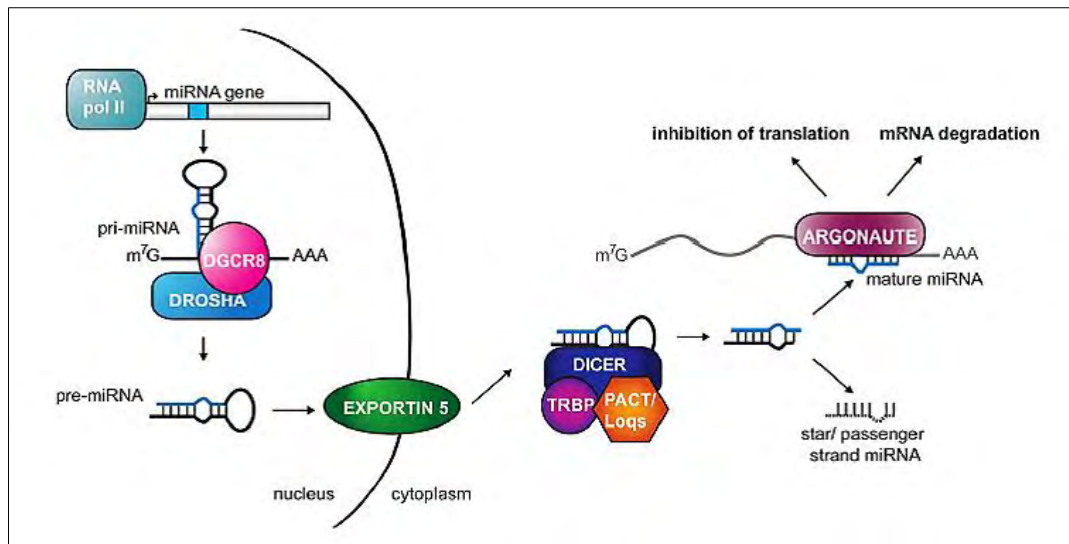


Figure 4.1. MicroRNA biogenesis pathway (Finnegan & Pasquinelli, 2013).

4.2. MicroRNAs as therapeutics

MiRNAs are involved in the control of the majority of cellular functions such as developmental transitions, organ morphology, bilateral symmetry, stress response, metabolic pathways, cell proliferation, apoptosis, even carcinogenesis and cancer progression (Hummel et al, 2010). An individual miRNA is capable of regulating the expression of more than one targeted mRNAs and each mRNA can be affected by multiple miRNAs (Cai et al., 2009). Moreover miRNAs seem to be expressed in a development- and tissue-specific manner (Zhao & Srivastava, 2007; Cai et al., 2009). All these properties along with the latest technologies have rendered miRNAs valuable diagnostic and therapeutic targets especially in cancer research.

The most wide spread application of miRNA therapeutics relies on the prediction and potential modification of the cells' response to anticancer therapy (Hummel et al., 2010). Three main approaches have been described. Specific alterations in miRNA expression profiles of tumor cells could be indicative of their sensitivity or resistance to several treatments and therefore predict the outcome before the initiation of the treatment. Additionally changes in the expression that occur during therapy might be a useful tool to assess the success of the therapy. The Up- or down – regulation of

individual miRNAs can also be useful to enhance the sensitivity of cells to chemotherapy (Xue et al., 2016; Li et al., 2017).

MicroRNA mimic molecules have been designed to modify the expression or action of specific miRNAs. MiRNA mimics are small chemically modified RNA duplexes that can be loaded into RISC and provoke the downstream inhibition of the targeted mRNAs as would happen with endogenous miRNAs. Similarly miRNA inhibitor molecules have been designed to achieve the opposite purpose, in other words to impede the synthesis or action of specific endogenous miRNAs (Shah et al., 2016). A popular type of synthetic miRNA inhibitors, the antagomirs, can enter the cells and bind to the targeted miRNA, blocking its interaction with complementary transcripts (Luck, Muljo, & Collins, 2015).

Numerous studies have validated the efficiency of these approaches both in *in vitro* and *in vivo* models (Shah et al., 2016). A crucial challenge that has to be overcome is the selection of the appropriate delivery mechanism. The carrier has to be efficient in transfecting the cells and delivering its cargo and specific in order to avoid off-target toxicity. For this purpose several types of carriers have been evaluated including viral systems, nanoparticles and exosome-shaped carriers (Luck et al., 2015) (Fig. 4.2.).

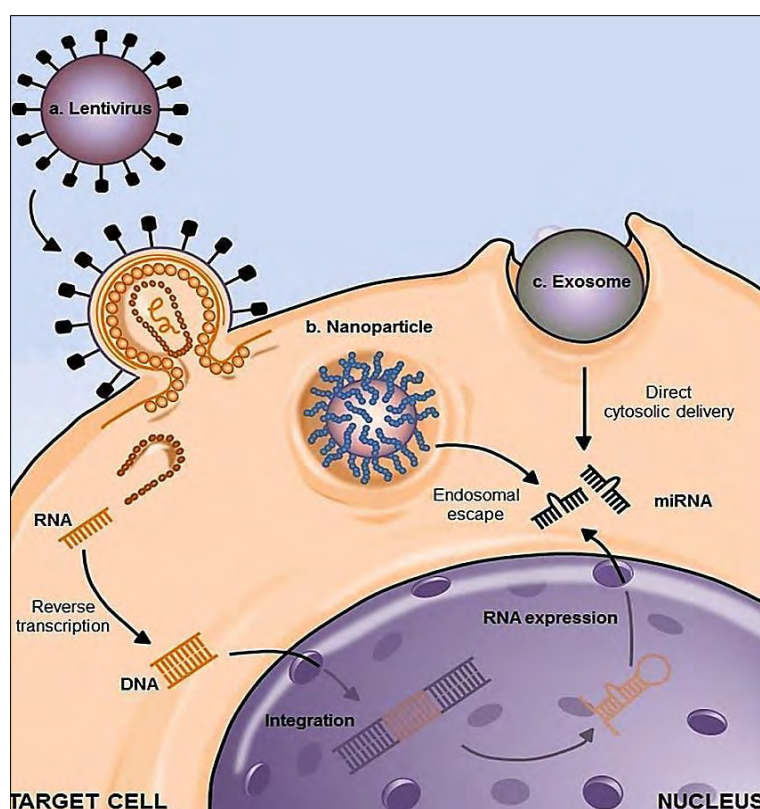


Figure 4.2. Delivery systems currently used in miRNA therapeutics include: a. lentiviruses, b. nanoparticles, c. exosomes (adapted from: Luck et al., 2015).

CHAPTER 5 – FERTILITY PRESERVATION IN CANCER PATIENTS

5.1. Fertility preservation strategies in female cancer patients

As it has been mentioned before, anticancer therapies can be compromising to the patients' fertility. However, several options are available and can be proposed for fertility preservation.

The most effective and well-established methods include oocyte and embryo cryopreservation. Both methods are commonly used nowadays and share similar success rates in IVF outcomes (Howards et al., 2014). The main disadvantage of these approaches is that they require about two weeks of ovarian stimulation prior to oocyte retrieval and this period of time as well as the administration of hormones might interfere with the patient's treatment (Sugishita & Suzuki, 2018). Moreover issues with the stimulation protocol might occur, including poor-response and hyper-stimulation syndrome (Guzy & Demeestere, 2017).

Alternative methods are ovarian tissue cryopreservation and in vitro maturation of immature oocytes. Actually, the only option currently available for prepubertal girls is ovarian tissue cryopreservation (Oktay et al., 2018). This method includes removal of the whole ovary or parts of it by laparoscopic surgery and storage at very low temperatures until the termination of the treatment. Re-implantation of the tissue is expected to restore the patient's fertility in a short period of time (Hamish et al., 2016). Even though this method is still considered experimental, its success cannot be underestimated as 86 live births have been reported so far worldwide (Sugishita & Suzuki, 2018). However, there are still technical difficulties to be overcome and the procedure should be considered as invasive and potentially risky since surgery is required (Hamish et al., 2016; Guzy & Demeestere, 2017).

Last a pharmacological strategy has been proposed using adjuvant drugs that can prevent the loss of the ovarian reserve. Ferto-protective adjuvant therapy is a term used to describe any pharmacological adjuvant administered during or prior to chemotherapy that can prevent the loss of the ovarian reserve (Mahajan, 2015). Although this approach seems very promising only few drugs have been proposed so far and only GnRH analogues are currently at clinical use (Oktay et al., 2018). Both GnRH agonists and antagonists have been examined over the past years with

contradictive results. Some research groups reported significant success of the method while others observed no conclusive evidence of GnRH analogues' protective effect (Meirow et al., 2004; Kishk & Mohammed Ali, 2013; Demeestere et al., 2013). As a general conclusion, adjuvant therapy with GnRH analogues is not among the recommended methods of fertility preservation but it can be considered in some cases, taking into account the limitations, controversy and potential side effects (Oktay et al., 2018).

5.2. MicroRNAs as a potential fertility preservation strategy

Even though the main application of miRNAs in therapeutics aims to enhance the sensitivity of cancer cells to chemotherapy, they may also be helpful in maintaining healthy cells that are affected by chemotherapy (Xue et al., 2016; Li et al., 2017). MiRNAs have shown high potential to regulate the expression of genes with fundamental functions in the cells such as proliferation, DNA damage response and apoptosis. Moreover miRNAs are capable of modulating their expression as a response to changes in the cell environment, including the exposure to cytotoxic agents (Bordinhao et al., 2016; Evert et al., 2018). By studying the role of miRNAs in the follicles' response to chemotherapy and targeting specific miRNAs it is possible to develop a therapy that can keep the follicles quiescent and healthy and therefore protect the female fertility.

CHAPTER 6 – GOAL OF THE STUDY

This study is based on the observation that miRNAs change their expression profiles during exposure to chemotherapy and the aim is to identify miRNAs with ovarian protective properties during oncological treatments. Previous studies have proven that miR-10a is significantly down-regulated in PND3 mouse ovaries after exposure to 4-HC for 1-24h. The goal of the present study was to restore the levels of miR-10a by transfecting the ovary with a mir-10a mimic and subsequently to determine the effect of the transfection on the primordial follicle activation, proliferation and apoptosis.

CHAPTER 7 – MATERIALS AND METHODS

7.1. Animal model

Female C57blxCBAF1 hybrid mice at 3-days old (PND3) were used in all the experiments (Fig. 7.1.). Animals were maintained under standard controlled light and temperature conditions and provided with food and water ad libitum. All the experimental procedures were approved by the Animal Ethics Committee of the Medicine Faculty at the Université Libre de Bruxelles.



Figure 7.1. Female PND3 C57bl x CBAF1 hybrid mice.

7.2. Ovarian culture

Female PND3 mice were sacrificed by decapitation. The ovaries were dissected and transferred to Leibovitz's Medium L-15 (Gibco LifeTechnology©) supplemented with fetal bovine serum (FBS 10%), 1 mg/ml streptomycin and 6 mg/ml penicillin G (Sigma©). Under the microscope, the PND3 ovaries were carefully cleaned and the surrounded tissues were trimmed away using insulin needles. Each ovary was cultured on a polycarbonate insert using Corning® 24 Well Tissue Culture-Treated Plates. Four conditions were created: culture alone (control), chemotherapy exposure alone (4-HC /20μM/1 hour), miR-10a-mimic alone, and miR10a-mimic + chemotherapy exposure (4-HC /20μM/1 hour). For the control condition, the ovaries were cultured in 400μl of Opti-MEM reduced-serum medium (Invitrogen, Life Technologies)

supplemented with ascorbic acid (50 µg/ml), human transferrin (27.5 µg/ml), penicillin G (5 IU/ml) and streptomycin sulphate (3.7 IU/ml) (Sigma©).

For the in vitro transfection of PND3 ovaries with miR-10a mimic, (mirVana® miRNA mimic, Life Technologies Europe BV), we used the Lipofectamine RNAiMAX transfection reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions with some modifications as it will be described. The transfection mix was prepared using 300pmol of miRNA-mimic and 20µl of Lipofectamine RNAiMax in 400µl of Opti-MEM reduced-serum medium. The transfection mix was incubated for 15 minutes at room temperature to form the Lipofectamine-miRNA complexes prior to transfection into ovaries. Then, the ovaries were cultured in 400µl of the transfection mix for 2 days at 37°C in a humidified incubator with 5% CO₂.

After 2 days in culture the ovaries (with or without miRNA-mimic transfection), were exposed to 4-HC (20µM) or not for one additional hour before processing for RNA extraction or histological assessment.

Table 7.1. The PND3 ovaries were cultured under four different culture conditions.

Sample Name	Culture Condition
Control	400 µlOPTIMEM
Chemo	400 µlOPTIMEM+ (4-HC /20µM/1 hour)
miR-10a	400 µlTransfection mix
Chemo + miR-10a mimic	400 µl Transfection mix + (4-HC /20µM/1 hour)

7.3. Individual TaqMan® Gene Expression Assays

The quantification of the miRNA expression levels was performed by qPCR with individual TaqMan® primer-probes for miR-10a and control genes U6 and snoR202. Briefly, 1µl from the diluted pre-amplified product was combined with 1µl of TaqMan™ MicroRNA Assays (20x) (Applied Biosystems™) and 10µl of

TaqMan™ PCR Master Mix (Applied Biosystems™) in a final volume of 20µl. All reactions were run in triplicates.

7.4. miRNA isolation and cDNA synthesis

Total RNA enriched in miRNAs and other small non-coding RNAs was isolated from PND3 ovaries using the ReliaPrep™ miRNA Cell and Tissue Miniprep System (Promega) according to the manufacturer's instructions, including an homogenization step with a pestle mixer to increase the effectiveness of the extraction. The system also incorporates a DNase treatment step to minimize the possibility of genomic DNA contamination. The quantity and purity of RNA samples was determined using NanoDrop™ spectrophotometer (Thermo Scientific) while for the RNA integrity, the RNA Integrity Number (RIN) was measured using the Agilent 2100 Bioanalyzer system. The samples were stored at –80°C or processed directly to cDNA synthesis. Samples were reverse transcribed into cDNA using the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems™) and multiplexed Megaplex™ RT Primers (Applied Biosystems™) according to the manufacturer's instructions. In order to increase the sensitivity, a step of pre-amplification was performed using 2.5µl from the cDNA product and the TaqMan® PreAmp kit (Applied Biosystems™) and Megaplex™ PreAmp Primers (Applied Biosystems™) according to the manufacturer's instructions. The pre-amplified products were diluted in final volume of 100µl TE buffer (pH8).

7.5. RNA extraction

Specific mRNA-targets expression levels were evaluated by qRT-PCR. Cultured ovaries that had been treated for 2 days under culture alone (control), chemotherapy exposure alone (4-HC/ 20µM/ 1 hour), transfection alone, or transfection + chemotherapy exposure (4-HC/ 20µM/ 1 hour) conditions were collected for mRNA

expression analysis. Each collection of PND3 ovaries was subjected to total RNA extraction using the ReliaPrep™ RNA Tissue Miniprep System (Promega) according to manufacturer's guidelines. The tissue homogenization steps were facilitated by the use of pestle mixer. To quantify the extracted RNA and assess its purity we used a NanoDrop™ Spectrophotometer (Thermo Scientific). The total RNA was stored at –80°C prior to use.

7.6. *In silico* gene expression analysis

We used the TargetScan database (targetscan.org) and the MirTarBase (mirtarbase.mbc.nctu.edu.tw) for the *in silico* prediction and identification of the genes targeted by mmu-miR-10a. A list of predicted and validated gene-targets was submitted to DAVID database (david.ncifcrf.gov) for annotation, visualization, and integrated discovery for pathway enrichment analysis.

7.7. qRT – PCR

Reverse transcription reactions were performed using 200ng of total RNA and random primers according to GoScript™ Reverse Transcriptase Promega® kit. For the quantification of gene expression, 2ng of CDNA were used in a qPCR reaction with SYBR® Green Master mix (Applied Biosystems™) and 10μM of gene-specific forward and reverse primers (Eurogentec). Primer sequences are listed in the table (Table 7.2.). Individual samples were analysed in duplicates and the gene RPL19 was used for data normalization. The expression level of each gene was calculated by the comparative Ct method (ΔCt) and the fold change was calculated by the equation $2^{-\Delta Ct}$. The final fold change was expressed in terms of geometric mean.

Table 7.2. Targeted genes and their primers for the qPCR.

Targeted gene symbol	Official Name by Mouse Genome Informatics (informatics.jax.org)	Primers' sequence (Forward-Reverse)
PTEN	phosphatase and tensin homolog	F: TTC-ACG-TCC-TAC-CCC-TTT-GC R: GGT-CCA-GAG-CCC-AGG-TAG-AA
FasI	Fas ligand (TNF superfamily, member 6)	F: AGA-CAG-AAC-CGC-AAG-ACA-GG R: CCC-ACA-GCC-TTG-AAA-CCG-AT
Casp3	Caspase 3	F: CTG-GGT-TGT-ACT-TCG-TGT-ATT-CC R: TGT-CCA-GTA-GTG-CAG-TAG-TTC-AA
Bcl2	B cell leukemia/lymphoma 2	F: ATC-ATT-CAG-GCC-TGC-CGG-GGT R: GGA-CTG-GAT-GAA-CCA-CGA-CCC-G
Bax	BCL2-associated X protein	F: TTG-TAA-TTC-ATC-TGC-CGC-CG R: AGG-GTT-TCC-AGA-TTG-GGT-CC
Irak1	interleukin-1 receptor-associated kinase 1	F: CAA-GAC-CAG-GGT-GGC-TGG-GAA-G R: AGA-CAC-AGT-CCA-AGG-CAG-TGG-GA
Smad1	SMAD family member 1	F: CAG-AGG-TGG-AAC-AGC-TAT-CAA-G R: CAT-TGG-GCA-AGA-AGC-CAT-AAA-C
Bmp4	bone morphogenetic protein 4	F: GGT-TCC-AAG-CAG-AAG-GAG-GT R: ATT-GTA-CTC-GCT-GTG-CCT-CG
Kitl	kit ligand	F: CCC-GGA-AGC-TAG-GTG-AGT-TC R: GCA-GGC-TCC-AAG-ACG-GTT-TA
Ccnt2	cyclin T2	F: TGGTGGCAAATCTTCCAAAT R: TTCTTCGGTGCGTTTCTTC
Stat3	signal transducer and activator of transcription 3	F: CTA-GAC-GGG-AAT-GTG-GTG-CC R: GTA-GTG-TCA-GCG-TTC-CTG-AAG-A
Hmgb3 (Hmg2a)	high mobility group box 3	F: CAA-GAG-GCA-GAC-CTA-GGA-AAT-G R: GAT-CCA-ACT-GAT-GCT-GAG-GTA-G
Mtor	mechanistic target of rapamycin kinase	F: ATC-CAG-ACC-CGT-AAC-CTC-CA R: ACA-GGC-ACC-CAT-CCA-ATC-TG
Rptor	regulatory associated protein of MTOR	F: AAT-GCT-GGC-CTC-ATC-GTC-AA R: CAT-CTG-GGC-AAG-TGG-ATG-GT
Rpl19	ribosomal protein L19	F: GAA-AGG-TGC-TTC-CGA-TTC-CA R: TGA-TCG-CTT-GAT-GCA-AAT-CC

7.8. Statistical analysis

All statistical analyses were performed on the GraphPad Prism software. At least three biological replicates were performed for every experiment in this study and all values represented as the mean or geometric-mean \pm standard error of the mean (s.e.m.). A paired t-test was performed for data following a normal distribution, while Mann Whitney U non-parametric test was used to analyze the data which was not normally distributed among groups. P-value < 0.05 was defined as a statistically significant difference.

7.9. Histology sample preparation

After 2 days of culture with or without treatments the ovaries were fixated in 4% paraformaldehyde for 2 hours at 4°C. The fixation of tissue samples aims to preserve them against decay and structural damage by terminating all ongoing biochemical reactions (Ganjali & Ganjali, 2013). Fixated tissue was then dehydrated and embedded in paraffin heated at 60°C. Samples were allowed to completely harden overnight and on the following day the tissue was cut in sections of 5 μ m by microtome.

7.10. Hematoxylin and Eosin Staining

Hematoxylin and Eosin (H/E) staining is a useful technique to recognize a variety of tissue samples and study the morphological changes in the tissue under different conditions. Hematoxylin has a deep blue/purple color and stains nucleic acids whereas Eosin is pink and stains proteins nonspecifically. Typically in a tissue sample the nuclei is stained blue while the cytoplasm and extracellular matrix show varying

degrees of pink staining (Fischer, Jacobson, Rose, & Zeller, 2008). For this staining, every fifth section was mounted on a slide and stained for assessing the follicular morphology.

7.11. TUNEL assay

Terminal deoxynucleotidyl Transferase (TdT) dUTP Nick End Labeling (TUNEL) is an assay used to detect apoptotic cells or cells undergoing DNA damage response in situ. TdT has the ability to label blunt ends of double-stranded DNA breaks by catalyzing the addition of labeled dUTPs to a 3'-hydroxyl termini of DNA ends hence, the degrading DNA can be visualized using immunohistochemical techniques (Kyrylkova et al., 2012).

The process can be summarized in five steps. The samples were deparaffinized and rehydrated. Next they were treated with QIAGEN Proteinase K solution in order to induce breaks in the cellular membrane and obtain access to the nucleic acids. A positive control was generated with DNase treatment. TUNEL Buffer and Enzyme Solution were then applied, as indicated by the manufacturer. Last, Hoechst was used for DNA staining. Sections were observed using a Leica DM 2000 fluorescent microscope: cells appeared blue due to the DNA staining and the apoptotic cells were stained red.

The image analysis was performed using ZEN 2.3 software on at least 3 randomly selected sections per ovary from 2 individual experiments. Hoechst and TUNEL positive cells were quantified considering a lower thresholding to exclude all autofluorescence signals. Then TUNEL cells were expressed as a percentage of total Hoechst positive cells per ovary.

7.12. Ki-67 immunostaining

The proliferation rates of the follicles were examined using Ki67 Immunostaining. Ki-67 is protein exclusively present in the nuclei of cells undergoing phases G1, S and

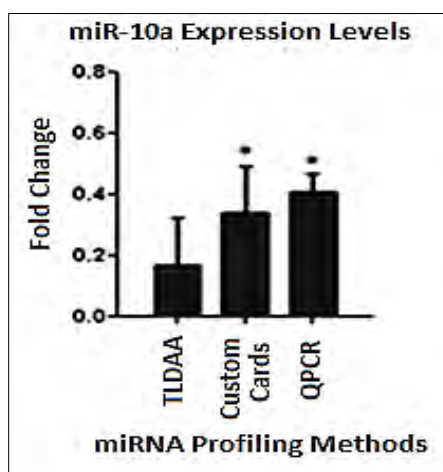
G2 of the cell cycle as well as mitosis. On the other hand, quiescent and resting cells in G0 do not to express the Ki-67 antigen (Scholzen & Gerdes, 2000).

Before immunostaining the ovarian sections were deparaffinized and rehydrated. Heat-mediated epitope retrieval was performed in citrate buffer (pH=6.0). Hydrogen peroxide 1% (Merck Millipore, Belgium) was used to block endogenous peroxidases. In order to avoid non-specific binding, Normal Goat Serum (NGS) was used as blocking solution before incubation with primary antibody (KI-67 mouse α -human 1:400, BD Bioscience, Belgium 1:1000, Cell Signaling, Netherlands) overnight at 4°C. At the next step, the sections were incubated with secondary biotinylated goat anti-mouse antibody (1:300, Vector Laboratories, UK), followed by treatment with an ABC kit (Vectastain Elite ABC systems, Vector Laboratories, UK). Diaminobenzidine (DAB Peroxidase Substrate Kit, Vector Laboratories, UK) was used for reaction revelation while haematoxylin was used for background staining. Last, the sections were observed in Leica DM 2000 fluorescent microscope.

CHAPTER 8 – RESULTS

8.1. MiR-10a is significantly down-regulated after chemotherapy exposure in PND3 mouse ovaries

Previous study in our lab attempted to identify miRNAs involved in mechanisms of primordial follicle activation, DNA damage response and apoptosis during exposure to 4-HC, by studying the miRNA expression profiles in PND3 mouse ovaries. The study concluded that miRNAs rapidly respond to the alterations of their microenvironment by changing their expression profiles. Among the most affected miRNAs, miR-10a was significantly down-regulated after 1-24h of exposure to 4-HC (Fig. 8.1.). Based in these results we decided to further investigate the role of mir-10a in the ovary by performing miRNA-mimic transfection experiments *in vitro*.



miRNA Profiling Methods	Fold Change TLDA	Fold Change Custom Cards	Fold Change QPCR Assays
miR-10a	0.16	0.34	0.40

Figure 8.1. miR-10a expression profiling by TaqMan® Low Density Arrays and validation of its expression levels by TaqMan® Custom Cards and individual TaqMan® Assays. MiR-10a showed a down-regulated profile through all the miRNA profiling methods after 1h exposure to 4-HC/20μM.

8.2. Pathway Enrichment Analysis of Genes Targeted by miR-10a

In order to identify which pathways are regulated by miR-10a, we performed functional annotation clustering on DAVID database for the predicted (TargetScan) and validated miR-10a targeted genes (miRTarBase) (Fig. 8.2.A. and B.).

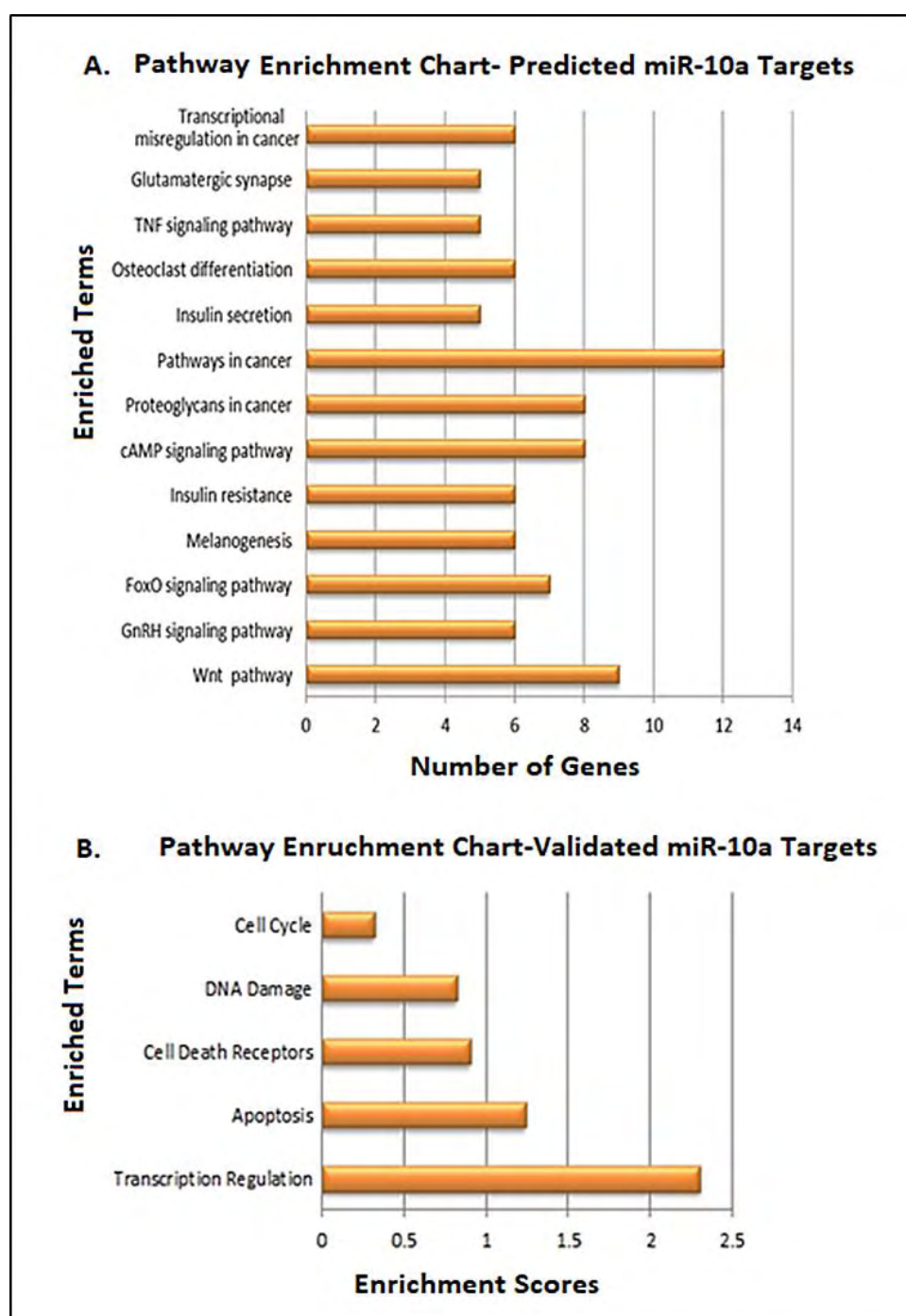


Figure 8.2. Functional annotation on predicted (A) and validated (B) targets of miR-10a. The targeted genes are implicated in DNA damage response, apoptosis and cell cycle regulation.

8.3. Evaluation of the transfection's efficiency

The transfection of PND3 ovaries *in vitro* was performed by Lipofectamine, a liposome-based delivery system. This delivery system has been tested in cell lines but there is no established protocol for the transfection of whole organs *in vitro* such as PND3 ovaries. However, in previous experiments in our lab, a transfection protocol was created based on different concentrations of the Lipofectamine and the mimic-miRNA molecules, while the transfection's efficiency was verified after transfection with fluorescent label dsRNA molecule. After 48 hours in culture with the transfection mix (Lipofectamine + Alexafluor labeled dsRNA), the fluorescent signal was detected in every ovarian sections, indicating that the transfection is successful (data is not showed). In the case of miR-10a mimic delivery, we followed the same protocol while the transfection's efficiency was verified after quantification of miR-10a expression levels. By comparison with non-transfected ovaries (control), we found that the levels of miR-10a were significantly increased in miR-10a transfected group ($p < 0.05$) (Fig. 8.3.).

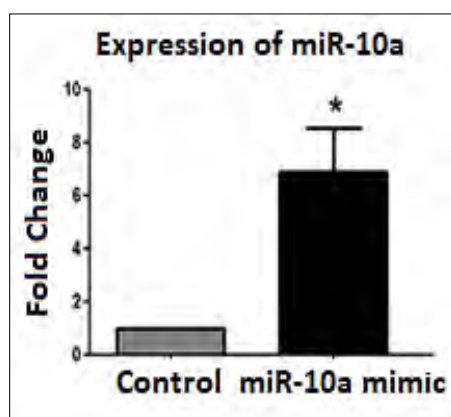


Figure 8.3. Quantification of miR-10a expression levels by qPCR. The fold change of miR-10a is significantly higher in the miR-10a mimic group compared to the control (*p= 0.037, N=3).

8.4. Assessment of the levels of apoptosis

8.4.1. Evaluation of apoptosis by TUNEL Assay

The apoptosis levels were evaluated by TUNEL and quantified. The PND3 ovarian sections (5µm) show nuclear labeling with blue (Hoechst) whereas the apoptotic cells are stained red. In the merged images, we can observe that the number of apoptotic cells in the PND3 ovaries is increased after exposing them to chemotherapy. However, the quantification of TUNEL positive cells does not indicate that the transfection with miR-10a has a protective role in PND3 ovaries after exposure to 4-HC/20µM for 1 hour (Fig. 8.4. A & B).

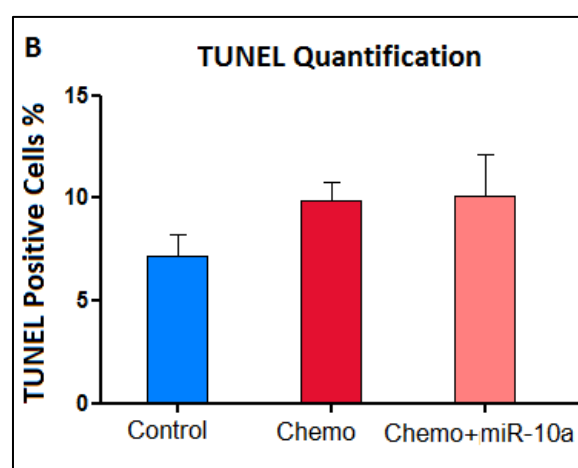
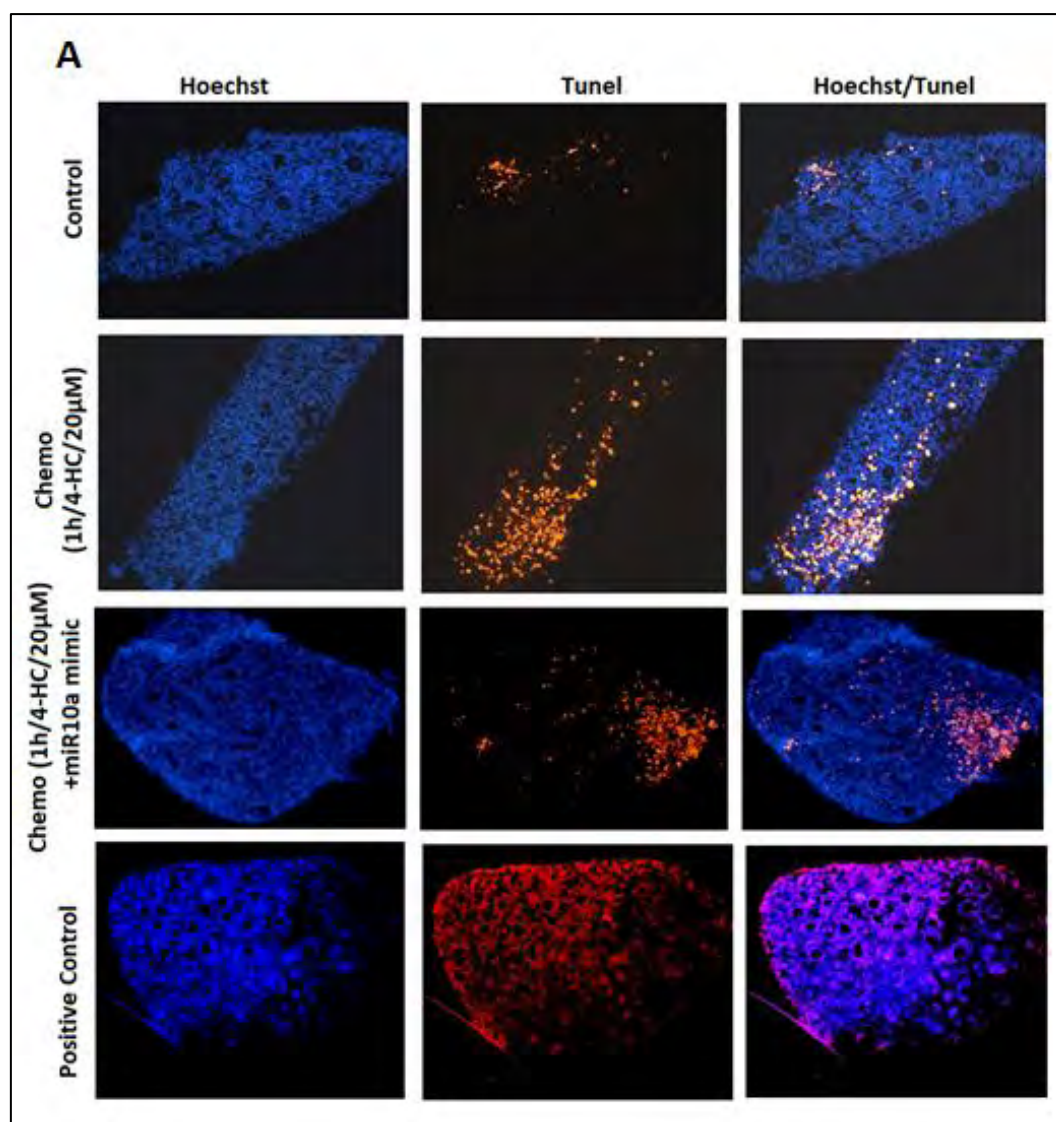


Figure 8.4.A. Representative images of PND3 ovaries after 2 days of culture under different conditions. PND3 ovarian sections (5μ) show nuclear labelling with Hoechst (blue) and apoptotic cells (red). **B.** Quantification of TUNEL positive cells (N=2).

8.4.2. Evaluation of apoptosis-related genes expression by qPCR

A number of genes involved in apoptosis were selected for studying their expression under chemotherapy exposure and transfection with miR-10a mimic. The expression analysis was performed in pairs of control/ chemo, control/miR-10a mimic and chemo/chemo+miR-10a mimic. We found that after 1h exposure to 4-HC, the expression of BAX is significantly up-regulated in the treated group compared to control ($p<0.05$) (Fig. 8.5.). However, the transfection with miR-10a could not suppress the expression of this gene. There were no significant changes in genes expression after comparison between control and miR-10a mimic transfected groups. However, the genes BCL2 and STAT3 were significantly up-regulated in chemo+miR-10a group while the IRAK1 was significantly down-regulated compared to chemotherapy exposed only ($*p<0.05$) (Fig. 8.6. A & B).

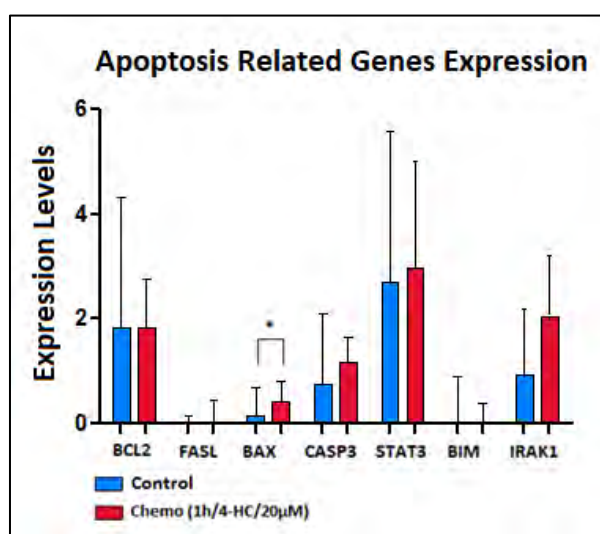


Figure 8.5. Expression levels of genes related to apoptosis in control and chemotherapy exposed groups (1h/4-HC/20μM). BAX is significantly up-regulated in the chemo group compared to control ($p=0.043$, $N=7$). The error bars represent the standard error, paired t-test, $*p<0.05$.

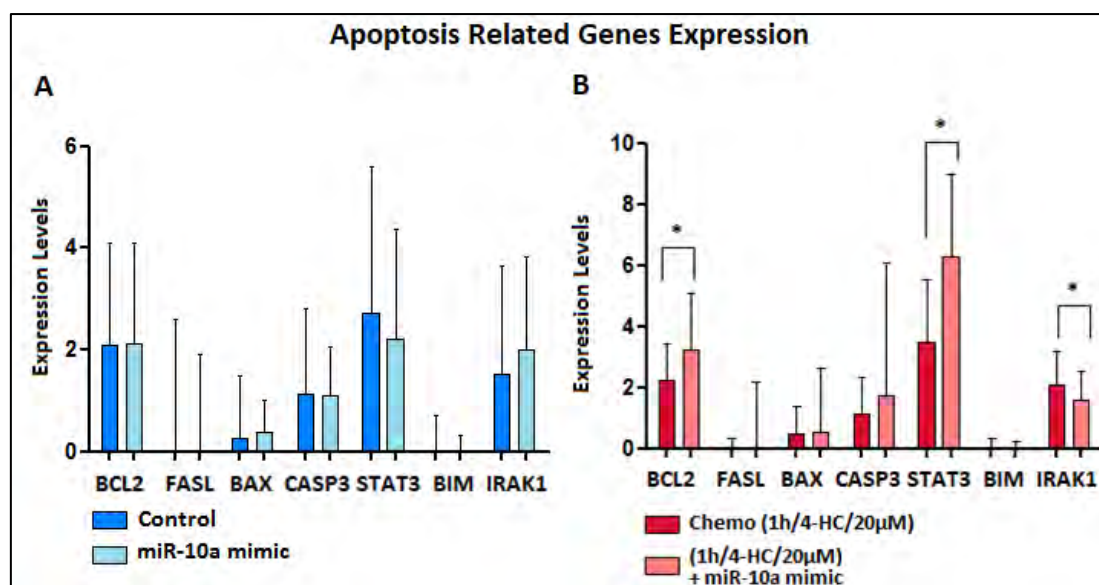


Figure 8.6. Expression levels of genes related to apoptosis in the different conditions. The graphs present the genes expression levels in pairs of groups: **A.** Control/miR-10a mimic, **B.** Chemo/Chemo +miR10a mimic. BCL2 ($p=0.049$, $N=9$) and STAT3 ($p=0.032$, $N=9$) were significantly up-regulated in chemo+miR-10a group while IRAK1 was significantly down-regulated compared to chemotherapy exposed only ($p=0.009$, $N=8$). The error bars represent the standard error, paired t-test, $*p<0.05$.

8.5. Assessment of the follicle morphology and proliferation

8.5.1. Evaluation of the follicle morphology and proliferation by H/E and Ki-67 staining

To assess the transfection's impact on the follicle morphology, number and maturation stage we performed histological studies by Haematoxylin & Eosin staining and Ki-67 immunostaining on ovarian sections ($5\mu\text{m}$) (Fig. 8.7. A & B). Primordial and growing follicles can be identified in the ovaries after 2 days of culture.

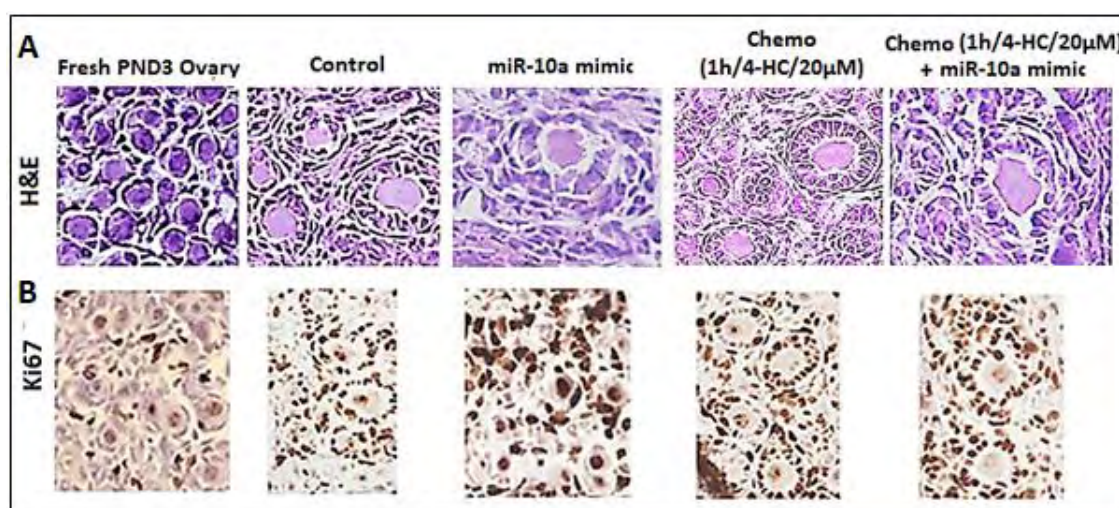


Figure 8.7.A. Histological studies on ovarian sections cultured under different conditions. Hematoxylin & Eosin staining for the evaluation of the follicle morphology. **B.** Assessment of proliferation levels by Ki-67 immunostaining. The marker Ki-67 is stained in brown colour.

8.5.2. Evaluation of proliferation-related genes expression by qPCR

The expression levels of genes related to proliferation were also evaluated by qPCR. The gene PTEN is significantly down-regulated after transfection in both miR-10a mimic ($p=0.045$) and chemo+ miR-10a ($p=0.036$) groups compared to their controls. (Fig. 8.8.). PTEN is a validated target of miR-10a (Zeng & Li, 2014; Yu et al., 2015) and its down-regulated profile after miR-10a up-regulation indicates that the transfection is efficient. However, the impact on follicle activation due to PTEN down-regulation has to be examined further. Moreover, the gene Cyclin T2 (CCNT2) is significantly up-regulated in chemo+miR-10a group compared to chemo ($p<0.05$). A controversial expression is detected in the case of RPTOR. This gene was significantly down-regulated after transfection in miR-10a mimic ($p<0.05$) but in chemo+ miR-10a mimic group it was up-regulated compared to its control ($p<0.05$). RPTOR is not normally targeted by miR-10a, but is involved in follicle activation signalling. As it is not obvious that there is a direct influence on follicle activation after transfection, it will be interesting to study more the possible long-term impact on follicles due these gene expression changes. Last, there is no indication that

transfection with miR-10a affects other genes like KITL or mTOR which have also been characterised to play an important role in follicle development.

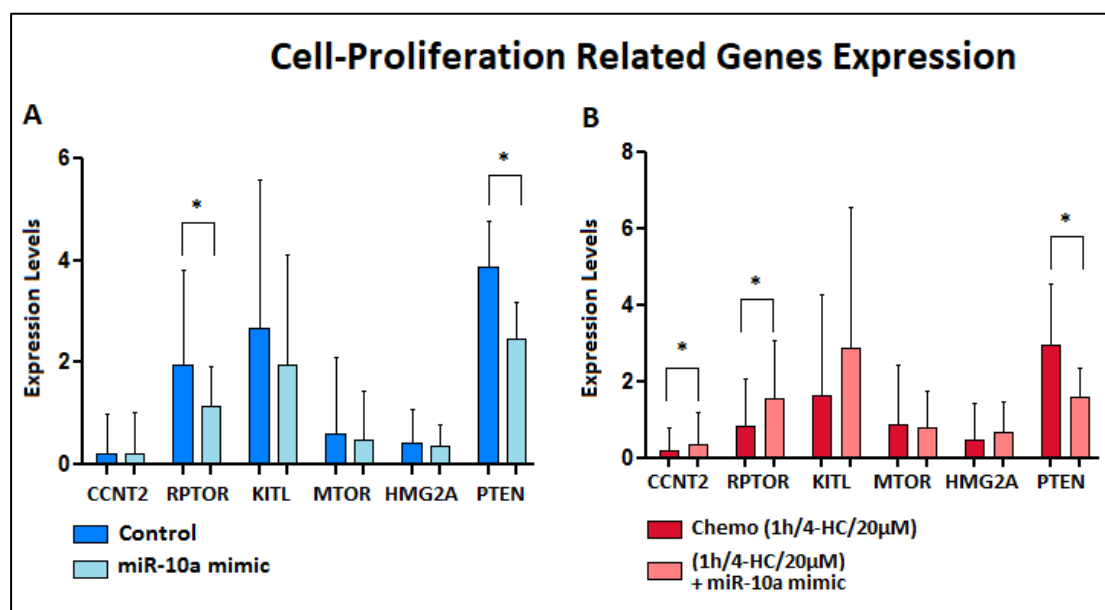


Figure 8.8. Expression levels of genes related to cell proliferation in different conditions. The graphs represent the genes expression levels in pairs of groups: A) Control/miR-10a mimic, B) Chemo (1h/4-HC/20μM)/ Chemo (1h/4-HC/20μM) +miR10a mimic. The gene RPTOR is significantly down-regulated after transfection in miR-10a mimic (*p=0.032, N= 8) but is up-regulated in chemo+ mir-10a (p= 0,039 N=8) group compared to their controls. PTEN is significantly down-regulated in both miR-10a mimic (*p=0.045, N= 8) and chemo+ mir-10a (*p= 0.036 N=7) groups. CCNT2 is significantly up-regulated in chemo+ mir-10a (*p=0.038, N=9). The error bars represent the standard error, paired t-test, *p<0.05.

8.6. The impact of miR-10a on TGF-β pathway

In previous study it has been shown that there is a regulatory loop between miR-10 family members and TGF-β members in granulosa cells (Jiajie, Yanzhou et al., 2017). Moreover, the importance of the TGF-β pathway during folliculogenesis, has been proven (Trombly, Woodruff, & Mayo, 2009; Myers & Pangas, 2010). Hence, we selected to quantify the expression levels of TGF-β genes such as BMP4 and

SMAD1. We found that after chemotherapy exposure for 1h (4-HC/20 μ M) the levels of SMAD1 seem increased compared to control, but we did not detect any decrease in the expression levels after transfection with miR-10a mimic in control or chemotherapy-exposed groups. As for the expression of BMP4, this gene has been found to be lowly expressed in PND3 ovaries and its expression was only up-regulated in chemo+ miR-10a groups but the result was not significant (*p=0.053, N=9) (Figure 8.9.). These controversial results cannot confirm that miR-10a acts as an inhibitor of the expression of BMP4 and SMAD1 in PND3 ovaries indicating that the cell type and the different culture conditions may affect these interactions and further studies must be conducted in order to draw a solid conclusion about the genes' interactions.

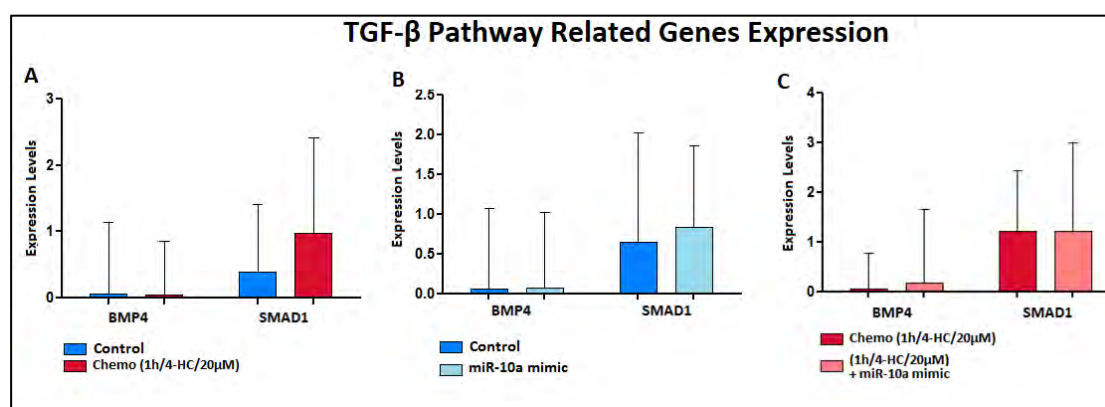


Figure 8.9. Expression levels of genes related to TGF- β signaling pathway in different conditions. The graphs present the genes expression levels in pairs of groups: A) Control/ Chemo, B) Control/miR-10a mimic, C) Chemo/Chemo+miR-10a mimic. The expression of BMP4 does not change under different conditions while the SMAD1 seems to be increased after exposure to chemotherapy or transfection with miR-10a but the results are not significant for *p<0.05 after t-test paired analysis in at least 7 experiments.

CHAPTER 9 – DISCUSSION

The present study is based on the observation that miRNAs respond to the changes of their microenvironment as they alter their expression profile during exposure to chemotherapeutic agents (Bordinhao et al., 2016; Evert et al., 2018). This miRNAs' characteristic may be useful in minimizing chemotherapeutic side-effects like ovarian toxicity. MiRNAs are tiny molecules which regulate the function of big networks of genes implicated in pathways that can be triggered by chemotherapy, such as apoptosis, DNA damage response and follicle activation. Hence, it is very interesting to study the potential of miRNAs in reducing off-target toxicity and to identify if some of them have ovarian protective properties. In this study, miR-10a was chosen for functional analysis after it was found to be down-regulated in PND3 ovaries exposed to 4-HC (1-24 hours).

Given that miR-10a was down-regulated in PND3 ovaries, we used a liposome-based delivery system to restore its function in PND3 ovaries. The transfection was proven to be successful as the levels of miR-10a were significantly increased compared to control ovaries. Then, we also identified that the gene PTEN, which is an experimentally validated target of miR-10a, was down-regulated after miR-10a mimic transfection which further confirms the transfection's efficiency (Zeng & Li, 2014; Yu et al., 2015).

The effect of miR-10 mimic transfection on apoptosis levels was evaluated by TUNEL Assay and qPCR for the selected apoptosis-related genes. BAX, which has a key role in apoptosis induction, was found to be up-regulated in PND3 ovaries after 1h chemotherapy exposure. However, its expression could not be down-regulated after miR-10a mimic transfection. Other genes like BCL2 and STAT3 (Signal Transducer and Activator of Transcription 3) were significantly up-regulated while IRAK1 (interleukin 1 receptor associated kinase 1) was significantly down-regulated after exposure to chemo+miR-10a mimic compared to chemo alone. These genes are important regulators of apoptosis and their altered profile may have an impact on the apoptotic processes (Tsujimoto, 1998; Levy & Lee, 2002; Bhattacharya, Ray, & Johnson, 2005; Rhyasen & Starczynowski, 2015). As these genes are not direct targets of miR-10a we can conclude that there is an indirect mode of regulation that has to be elucidated. Moreover, the preliminary results of TUNEL Assay did not

indicate a protective effect on miR-10a transfected ovaries after chemotherapy exposure but more experiments need to be carried out in order to draw a precise conclusion.

As for the impact of miR-10a mimic transfection on follicle activation and cell proliferation, we selected a number of genes involved in these pathways and we studied their expression by qPCR while we also performed histological assays to evaluate the follicle morphology and developmental stage. The expression of RPTOR (Regulatory Associated Protein of MTOR Complex 1) and PTEN (Phosphatase and Tensin Homolog) was down-regulated in miR-10a transfected ovaries. PTEN and RPTOR participate in PI3K/Akt/mTOR signalling pathway. PTEN is a negative regulator of PI3K (Reddy et al., 2008) while RPTOR regulates the function of mTORC1 (Sengupta, Peterson, & Sabatini, 2010). The down-regulated profiles of these genes may result in a balance of primordial follicle activation as the inhibition of PTEN induces the follicle activation, while the down-regulation of RPTOR has the contrary result. However, in the case of chemotherapy exposed-transfected ovaries, PTEN levels were decreased and RPTOR levels were increased, which means that there is a trend of inducing follicle activation. At the same pace, CCNT2, which is involved in cell proliferation, was up-regulated too. Even if they are not statistically significant, the profiles of genes involved in cell growth and development like BMP4 and SMAD1 were also up-regulated in chemo+miR-10a group. All these results indicate that miR-10a transfection may induce ovarian follicle activation under chemotherapy exposure. Nevertheless these results have to be confirmed after follicle counting.

Previous studies have attempted to elucidate the role of miR-10a in the ovary. In 2014, the team of Xiao et al. had observed a positive effect of Amniotic Fluid Stem Cells (AFSCs) in the preservation of primordial follicles after chemotherapy administration (Xiao et al., 2014). In 2016, the team found that miR-10a was the key-molecule in the anti-apoptotic effect of AFSCs on CTx-damaged granulosa cells (Xiao et al., 2016). On the contrary, a study by Jiajie et al. suggested that both miR-10a and miR-10b repressed proliferation and induce apoptosis in human, mouse and rat granulosa cells (Jiajie et al., 2017). The potential involvement of miR-10a in the control of ovarian cell proliferation and apoptosis in human has also been supported by Sirotkin et al. in 2010. The aim of this study was to identify specific miRNAs that participated in the regulation of these processes in cultured primary hGCs. A

significant decrease in the percentage of cells expressing PCNA and cyclin B1 markers of proliferation and a trend of increase in the percentage of cells expressing Bax, TdT and Caspase-3 markers of apoptosis were reported after transfection with miR-10a precursor molecule (Sirotkin et al., 2010).

Based on our results we support the role of miR-10a in apoptosis and cell proliferation in the ovary but do not suggest ovarian protection during chemotherapy exposure. We believe that more studies should be done regarding the genes which are targeted by miR-10a in order to elucidate the pathways regulated by this miRNA. Moreover, it is important to take into consideration that the miRNA expression is not only tissue-specific but also cell-specific and it can be altered in different developmental stages. Hence, before extrapolating about the role of miR-10a, we need to create a total panel of miR-10a expression under different conditions. Last, *in vivo* experiments should be done in order to confirm our results and to elucidate the role of miR-10a in the ovary after chemotherapy exposure.

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