The Effects of Bone Morphogenetic Protein 7 (BMP-7) on Steroidogenic Function and Luteinization Characteristics of Human Luteal Granulosa Cells

by

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Koç University

Graduate School of Health Sciences

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Date: August 19, 2021

To my father…

ABSTRACT

The Effects of Bone Morphogenetic Protein 7 (BMP-7) on Steroidogenic Function and Luteinization Characteristics of Human Luteal Granulosa Cells

Ceren Sultan Yıldız

Master of Science in Reproductive Biology

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BMP-7 is a member of the TGF-β superfamily which is mainly produced by theca cells in the ovary. It promotes the transition of primordial follicles into primary follicles, the growth of preantral and antral follicles. However, limited data is available regarding the role of this protein on the molecular luteal characteristics of granulosa cells after ovulation and luteinization processes. In this thesis, it was aimed to analyze the effects of BMP7 on steroidogenesis in human luteal granulosa cells (HLGCs) isolated from follicular fluids of 10 normo-responder IVF patients after the oocyte pick-up procedure. The expression of BMP receptors in these cells was validated with the qRT-PCR technique. BMP-7 treatment significantly down-regulated the expression of StAR which is the rate-limiting enzyme in steroidogenesis in immunoblotting, confocal images, and caused a substantial decrease in P_4 production in the luteal GCs. It did not cause any notable change in aromatase expression and E_2 production. On the other hand, hCG as a luteotropic hormone significantly up-regulated StAR expression and enhanced P_4 output whereas activin-A did the opposite effect and reversed luteinization phenotype. Reversal of luteinization and down-regulation of steroidogenesis with BMP-7 and other hormones with similar actions warrant further investigation to test their in-vivo effects in order to develop new strategies against ovarian hyperstimulation syndrome (OHSS) and this data can be used for clinical trials in the future.

Keywords: BMP-7, steroidogenesis, human luteinized granulosa cells, IVF

ÖZETÇE

Kemik Morfogenetik Protein 7'nin (BMP-7) İnsan Luteal Granüloza Hücrelerinin Steroidojenik Fonksiyonu ve Luteinizasyon Özellikleri Üzerine Etkileri

Ceren Sultan Yıldız

Üreme Biyolojisi, Yüksek Lisans

Ağustos 19, 2021

BMP-7, esas olarak yumurtalıktaki teka hücreleri tarafından üretilen TGF-β ailesinin bir üyesidir. Primordiyal foliküllerin primer foliküllere geçişini, preantral ve antral foliküllerin büyümesini destekler. Bununla birlikte, bu proteinin yumurtlama ve lüteinizasyon süreçlerinden sonra granüloza hücrelerinin moleküler luteal özellikleri üzerindeki rolüne ilişkin sınırlı veri mevcuttur. Bu çalışmada, oosit toplama işlemi sonrası normal yanıt veren 10 IVF hastasının foliküler sıvılarından izole edilen insan luteal granüloza hücrelerinde BMP7'nin steroidogenez üzerine etkilerinin incelemesi amaçlandı. Bu hücrelerde BMP reseptörlerinin ekspresyonu qRT-PCR tekniği ile doğrulandı. BMP-7, immünoblotlama ve mikroskopik görüntülerde steroidogenezde hız sınırlayıcı enzim olan StAR ekspresyonunu önemli ölçüde azalttı ve luteal granüloza hücrelerinin P4 üretiminde azalmaya neden oldu. Aromataz ve E_2 ifadesinde kayda değer bir değişikliğe neden olmadı. Öte yandan hCG luteotropik bir hormone olarak StAR ekspresyonunu önemli ölçüde arttırdı ve P4 üretimini arttırdı, aktivin-A ise ters etki yaptı. Luteinizasyonun tersine çevrilmesi ve steroidogenezin BMP-7 ile azalması ve benzer eylemlere sahip diğer hormonlar, yumurtalık hiperstimülasyon sendromuna (OHSS) karşı yeni stratejiler geliştirmek ve in-vivo etkilerini test etmek için daha fazla araştırmayı gerektirir ve bu veriler gelecekte klinik araştırmalar için kullanılabilir.

Anahtar kelimeler: BMP7, steroidogenez, insan luteinize olmuş granüloza hücreleri, IVF

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ABBREVIATIONS

GnRH	Gonadotropin releasing hormone
FSH	Follicle stimulating hormone
LH	Luteinizing hormone
hCG	Human chorionic gonadotropin
hMG	Human menopausal gonadotropin
IVF	In vitro fertilization
ICSI	Intracytoplasmic sperm injection
ART	Assisted reproductive technologies
TGF-β	Transforming growth factor-β
GDF9	Growth differentiation factor 9
PI3K	Phosphoinositide 3-kinases
COC	Cumulus-oophorus complex
CL	Corpus luteum
StAR	Steroidogenic acute regulatory protein
CYP11-A	Cytochrome P11-A
3β-HSD	3β-hydroxysteroid dehydrogenase
VEGF	Vascular endothelial growth factor
PKC	Protein kinase C
CREB	cAMP response element-binding protein
EGF	Epidermal growth factor
MAPK	Mitogen-activated protein kinase
CDK	Cyclin dependent kinase
SREBP	Sterol regulatory element-binding proteins
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A reductase
LDL	Low density lipoprotein
HDL	High density lipoprotein
LIPA	Acid lipase enzyme
NPC	Niemann-Pick type C protein
NCEH1	Neutral cholesterol ester hydrolase
LIPE	Hormone-stimulated lipase
BMP	Bone morphogenetic protein

AMH	Anti-Mullerian hormone
GDNF	Glial cell-derived neurotrophic factors
ALK	Activin receptor-like kinase
TβR2	TGF-β receptor 2
AMHR2	Anti-Mullerian hormone receptor 2
BMPR2	Bone morphogenetic protein receptor 2
ACVR2A	Activin receptor 2A
ACVR2B	Activin receptor 2B
ERK	Extracellular-signal-regulated kinase
JNK	c-Jun N-terminal kinase
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's phosphate-buffered saline
RIPA	Radio immunoprecipitation assay buffer
BSA	Bovine serum albumin
PVDF	Polyvinylidene difluoride
HRP	Horseradish peroxidase
ECL	Enhanced chemiluminescence
cDNA	Complementary DNA
PCR	Polymerase chain reaction
TAE	Tris-acetate-EDTA
PFA	Paraformaldehyde
HLGC	Human luteinized granulosa cells
XlAP	X-linked inhibitor of apoptosis protein
TAK1	TGF-β activated kinase 1
TAB1	TAK1 binding protein
NFkB	Nuclear factor kappa B
mTOR	Mammalian target of rapamycin

Chapter 1: **REVIEW OF LITERATURE**

1.1 Female Reproductive System

The reproductive or genital system is composed of the organs which are having a role in the sexual reproduction of the organism and ensures the production and joining of germ cells named egg or ovum in females and sperm in males. In addition to these functions, the female reproductive system is also responsible for nurturing and protecting the fertilized oocyte during gestation (Selvakumari & Selvakumari, 2018). The primary glands for reproductive cell production are named gonads. Although maturation of the gonads occurs at puberty, development of them begins in utero. In females, after approximately the age of 45, a decrease in fertility is observed, and eventually, it ends permanently; this permanent stop of the menstruation cycle is known as menopause (Davis et al., 2015).

The female reproductive system in humans is composed of several organs which are located in the body or outside of the body, known as internal and external genital organs. The external genital organs include labia majora, labia minora, clitoris, Bartholin's gland, vulva, mons pubis, and pudendal cleft (E. E. Jones, 2009). The main functions of these organs are to allow sperm to enter the body and defend the internal genital organs against the organisms that cause infections. Labia majora are large, lip-shaped organs enclosing and protecting the other genital structures. They contain some glands that secrete sweat and oil and are coated with hair after puberty. Labia minora are small, lip-shaped organs underlying the labia majora and surrounding the entrance of the vagina and urethra. Bartholin's glands are responsible for mucus production and are located near the vaginal opening. The clitoris is the part that labia minora come together, and it is small and sensitive to stimulation for erection (Gulia et al., 2017). The internal organs of the female reproductive system are the vagina, cervix, fallopian tubes, ovary, and uterus. The vagina is a tubular tract with a fibromuscular structure, and it is functioning in sexual intercourse and the birth of the child. It joins with the lower part of the uterus which is known as the cervix. The uterus is the structure in which the baby grows, it can easily expand while the size of the baby is increasing. In addition to this, the cervix part of the uterus allows the entering of the sperm and exiting of the menstrual blood. Ovaries are the gland located

on both sides of the uterus, they are oval-shaped and responsible for hormone secretion and egg production. Finally, fallopian tubes are located in the upper part of the uterus and are responsible for traveling the oocyte from the ovaries to the uterus. Moreover, fertilization of the oocyte by sperm takes place in fallopian tubes and the fertilized oocyte is transported to the uterus for implantation (E. E. Jones, 2009).

In females, the reproductive age starts at 11-16 years old known as puberty. After puberty, females have monthly cycles in which repeated hormonal activities are experienced. These monthly cycles are also called menstrual cycles since "menstru" indicates monthly (Zuckerman, 1950). Every month, a hormone is released from the pituitary gland and some of the oocytes are stimulated. One of them is selected and released to fallopian tubes from which it passes through and meets with sperm. If fertilization occurs, the oocyte goes to the uterus and embryonic development starts by attaching the oocyte to the endometrium. If fertilization does not occur, the endometrium and oocyte which is not ovulated will be shed off every month through menstruation (Graziottin & Gambini, 2015).

1.2 Infertility

Infertility is the inability to get pregnant after 1-year regular unprotected coitus which is a very common situation among couples in recent years. In case of infertility problems, in 35% of the couples, the problem is related to the woman; in 35% of the couples, the problem is related to man; in 20% of the couples, both of them have a problem and in 10% of the couples, the cause is unknown. Thanks to technological developments, 85-90% of infertility cases can be detected and 50-60% of them can be cured successfully (Medical & For, 2014).

When a couple is considered infertile, some examinations such as learning their medical histories about sexually transmitted diseases, sexual habits, medications, and surgeries are performed on them (Panchal & Nagori, 2014). The woman is questioned about her menstrual cycle, and if it is certain that the woman is ovulating, then her uterus and fallopian tubes are examined for blockage by using pelvic ultrasound or laparoscopy methods (Tao et al., 2018; Imaoka et al., 2003). Moreover, testing the reproductive tract for infections and the condition of the sperm in cervical mucus, observing if the

reproductive tract of a woman produces antibodies to the sperm of her partner or not, and evaluating the quality of semen are highly important in understanding the reason of infertility (You & Tested, 2021). In the case of men, the collection of sperm and analysis of the semen according to the guidelines about reference values for semen characteristics published by the World Health Organization is important. In addition to these guidelines, hormonal regulations, conditions of ducts and accessory glands, and immunological tests are examined to assess the reason for male infertility (Tamrakar & Bastakoti, 2019).

1.2.1 Female infertility

One of the factors that affect female infertility is disorders of ovulation such as polycystic ovary syndrome, hyperthyroidism, hypothyroidism, hyperprolactinemia which can be resulted from hormonal disorders and affect the release of the oocyte from ovaries (Unuane et al., 2011). Not only these syndromes but also disorders in eating habits of the female, getting cancer therapy by radiation or chemotherapy can cause some disorders in ovulation. Moreover, the secretion of gonadotropin-releasing hormone (GnRH) might not be enough for stimulating follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which control ovulation (S. Smith et al., 2003). Another factor that affects female infertility is abnormalities in the uterus or cervix such as tumors in the uterine wall or blocking of fallopian tubes which fail implantation in the uterus (Buck et al., 1997). Growing endometrial tissue outside of the uterus which is known as endometriosis can be another cause of female infertility since it can affect the functions of ovaries, fallopian tubes, or uterus (Sciences & York, 2012). In addition to these, other risk factors can cause infertility such as the age of the woman, using tobacco and alcohol, being overweight or underweight, and lack of exercise (S. Smith et al., 2003).

In table I, possible reasons and treatments for female infertility are shown. If a woman has a problem with GnRH secretion, GnRH stimulatory agonists can be administered in a small dose to induce ovulation. However, sometimes even though the secretion of GnRH is enough, the secretion of FSH or LH from the pituitary gland might not be enough for ovulation. In this case, an anti-estrogen oral drug called *clomiphene* (Clomid) is used to inhibit the negative feedback action of estrogen on FSH and LH secretion (Medical & For, 2014). Another treatment can be the administration of

gonadotropins, which contain FSH and LH, in case of insufficient FSH and LH secretion such as human menopausal gonadotropin (hMG). In the case of hyperprolactinemia, a drug inhibiting the prolactin secretion can be used and in the case of endometriosis, ovarian tumors, scars, or cysts, there should be a need for surgery to restore infertility (Baylis et al., 2014). For some cases, basic lifestyle regulations such as losing weight, not using an excessive amount of tobacco, alcohol, nicotine can restore fertility.

Table I. Possible reasons and treatment options of female infertility

Reason	**Treatment options**
Age	Donor egg
Too much exercise	Doing less exercise
Obesity or low body fat	Losing or gaining weight
GnRH insufficiency	Using GnRH agonists
Gonadotropin insufficiency	Using FSH, hMG, hCG, or clomiphene
Endocrine disorders	Surgery or hormonal therapy
Tubal blockage	Microsurgery, IVF
Endometriosis	Microsurgery, GnRH antagonist
Antibody production against sperm	Immunosuppressant drugs, ICSI
Pelvic inflammatory disease	Antibiotics

1.3 Assisted reproductive technologies

The infertile couples whose problems cannot be solved via the methods shown in table 1, still can have a baby with the help of assisted reproductive techniques (ARTs). ARTs can be used for storing the gametes, controlling fertilization of the gametes, testing the embryo for genetic abnormalities, monitoring embryonic stages etc. (Gardner et al., 2005). The process of introducing sperm into the reproductive tract is called artificial insemination (AI). In this case, the man places semen into a special cup by masturbating, and the sperms are washed and prepared by using specific techniques. In the end, these

sperms are introduced into the uterus for fertilization with the oocyte. Some drugs or hormones can be used for inducing ovulation of more than one oocyte to enhance the chance of fertilization (American Society for Reproductive Medicine, 2016).

1.3.1 In vitro fertilization (IVF)

"*in vitro*" means "in glass"; therefore, in vitro fertilization is a process during which sperms and oocytes are fertilized in a petri dish and the fertilized oocyte is placed into the uterus of the female. This technique is suitable for couples who have fallopian tube blockage or low numbers of sperms (Ledger, 2002).

There are several steps of the IVF procedure. The first one is the stimulation of ovaries and retrieval of eggs for which FSH analogs are used commonly. In order to increase the number of mature follicles in the ovary, these FSH analogs are used, and additionally, GnRH agonists or antagonists are used to prevent premature ovulation of the follicles in the ovary (Depalo et al., 2012). Using ultrasound, the growth of follicles is monitored and hormonal levels are controlled during follicular growth since at this stage, it is expected for estrogen levels to be high and progesterone levels to be low. After several follicles are matured, hCG is injected to mimic LH and cause maturation of the egg for triggering ovulation (Craciunas et al., 2018). At this stage, before ovulation, these oocytes are collected from the ovary with a long needle through the vaginal wall and it is important to collect more than one oocyte to increase the fertilization chance. The other step of the IVF procedure is fertilization and transfer of the embryo (Gurevich, 2020). The sperms should be ready at the time of fertilization as mentioned above and should be placed on the petri dish in which capacitation of sperms will be completed with the help of the fluid inside the dish (Palini et al., 2017). Then, the oocytes are added to the same dish and waited for fertilization to occur in 12-14 hours. By controlling and checking the two pronuclei stages of the zygotes, it is confirmed that fertilization is completed. After that, these embryos are placed into another dish for monitoring their further growth and in two days, their two-to-four cell stage should be seen (Plachot & Crozet, 1992). Furthermore, 5 days after fertilization the embryos should be seen as blastocysts and they are transferred into the uterus. Approximately in two weeks, it can be detected whether the embryo is implanted or not (Lupron et al., 2021).

Thanks to recent technologies, there are other methods developed for increasing the success rate of IVF. One of them is intracytoplasmic sperm injection (ICSI) in which, as the name indicates, sperm is injected into the oocyte directly with the help of a tiny needle (SA Board for People and Practices, 2019). In addition to ICSI, assisted hatching or zona drilling methods can be used to increase the chance of IVF. In this method, by removing a part of zona pellucida from the surface of the pre-embryo, the hatching may be facilitated (Update, 2021). In some infertility cases, the sperm of the man is directly taken from the testis with surgery and injected into the ovary by the ICSI method, then transferred into the uterus. For such cases, even immature sperms can be used for fertilization (Urology Care Foundation, 2019).

1.4 Follicular Development

Follicles are found inside the ovary and defined as small sacs filled with fluid. The most important feature of follicles is secreting hormones which have important roles during the menstrual cycle (Hartshorne et al., 2009) and there are different follicular stages during a lifetime.

Oocyte formation, also known as oogenesis, begins at 4 weeks of gestation with the formation of the first primordial germ cells within the epiblast and precedes the formation of follicles (Hayashi, 2018). As primordial germ cells migrate through the hindgut, they colonize in the gonadal ridges where they proliferate as oogonia into ovarian cysts. They contain precursor granulosa cells derived from gonadal ridges (Hummitzsch et al., 2013). As the oogonia continue to enlarge, primary oocytes will develop, and by the breakdown of ovarian cysts, primordial follicles will be formed. These primordial follicles are composed of primary oocytes surrounded by a layer of flattened granulosa cells and arrested at the diplotene stage of prophase I until puberty (Gura & Freiman, 2018). As the primordial follicles grow, granulosa cells will increase in number and their shapes will change. Flattened cells become cuboidal as the oocyte enlarges and this stage of follicles is called primary follicles. While oocyte grows further, granulosa cells establish multiple layers around the oocyte, and eventually by recruitment of progenitor cells found in the ovarian cortex, vascularized theca cells that surround granulosa cell layers are formed (Liu et al., 2015). In addition to these, the zona pellucida structure begins to form which

is a thin band of glycoproteins that separates follicular cells and the oocyte; also, it has specific proteins on its surface to which sperm will bind during fertilization (R. E. Jones & Lopez, 2014). At this stage, the follicle is called the secondary or preantral follicle. As the follicle continues to grow, the cavity in between oocyte and follicular cells is filled with proteoglycans and hyaluronan due to the movement of fluid that originates from the thecal vasculature (Rodgers & Irving-Rodgers, 2010) and this cavity will be called as antrum. When the first meiotic division has completed, a secondary oocyte has formed, and a large antrum is seen. The follicle is called Graafian or preovulatory at this stage (Figure 1).

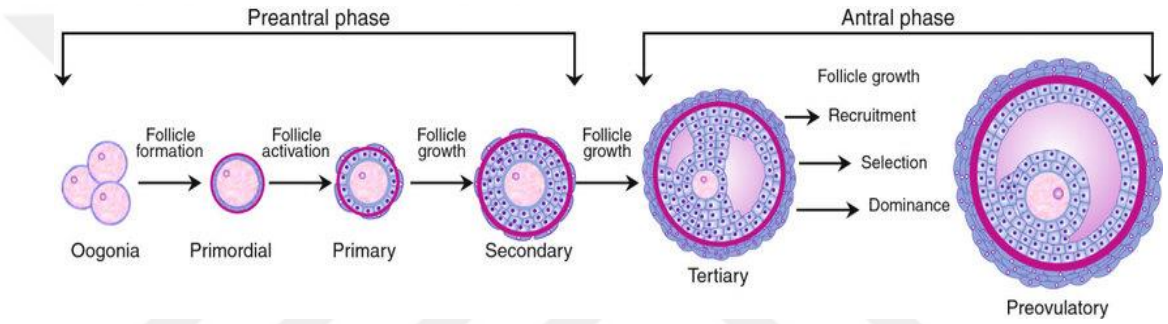

Figure 1: Schematic sequence of follicular development (Araújo et al., 2014)

1.4.1 Hormonal regulation of folliculogenesis

Several factors and mechanisms affect folliculogenesis; one of them is hormonal regulation of folliculogenesis which is controlled by the central nervous system, anterior pituitary, and ovary. Hypothalamus secretes gonadotropin-releasing hormone (GnRH) which is responsible for the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. These hormones are the main ones having a role in controlling folliculogenesis by acting on follicle cells (Singh & Krishna, 2010). FSH stimulates 15-20 preantral follicles at the beginning of each cycle after puberty. Although it is not necessary for primordial to primary follicle transition, without FSH these follicles become atretic and eventually die. In every cycle, one of these preantral follicles mature and only one oocyte is released while the others become atretic and degenerate (Gura & Freiman, 2018). In addition to stimulating preantral follicles, FSH also stimulates granulosa cell maturation around the oocyte, and proliferation of

granulosa cells is controlled by transforming growth factor β (TGF-β) family members especially growth differentiation factor 9 (GDF9) (McNatty et al., 1999). While granulosa cells increase in number and size, they begin to produce estrogen in response to FSH. On the other hand, LH stimulates theca cells to synthesize androgens such as androstenedione and testosterone which will act as estrogen precursors in granulosa cells and converted them to estrone and 17β-estradiol.

1.4.2 Molecular regulation of folliculogenesis

While terminal folliculogenesis is primarily controlled by FSH and LH, basal folliculogenesis is highly controlled by paracrine factors in between the oocyte and granulosa cells (Scaramuzzi et al., 2011). In recent years; with different genetic manipulations on mice such as a gene knock out, knock-in, overexpression, deletion the factors affecting the ovarian function are examined (Matzuk & Burns, 2012). It is found that most of the mechanisms are dependent on major cellular pathways which are the phosphatidylinositol 3 kinase (PI3K), the SMAD dependent pathway, and gonadotropin signaling cascades (Scaramuzzi et al., 2011). These pathways are important in the activation of the primordial follicle, primary to secondary follicle transition, follicular growth, and metabolism, the survival of growing follicle, terminal folliculogenesis until ovulation (Monniaux et al., 2018).

1.5 Ovulation

The term ovulation is known as the release of the egg from the ovary, and it is expected to occur around the middle of a menstrual cycle (28-day cycle). Many cell types should be involved in a proper ovulation process; these structures are theca, granulosa and cumulus oophorus cells, stromal tissue that surround the follicle and ovarian surface epithelial cells (Richards et al., 2015). Theca cell layer locates at the outermost layer of the follicle and consists of two types, theca interna and theca externa. In addition to supporting the follicular structure with the fibroblasts generating extracellular collagen, theca externa layer also provides nutrients and oxygen by creating a vascular network around the follicle (Magoffin, 2005). Theca and granulosa cells are separated from each other by a basal lamina which is thin, rich in collagen and laminin, and it helps granulosa

cells to attach to the surface especially the outer layer (mural) granulosa cells (Christensen et al., 2015). Other granulosa cells which surround the oocyte are called cumulus cells, and together with oocyte they form cumulus cell-oocyte complex (COC), and they are interconnected by connexin 43 composed gap junctions. Zona pellucida structure which separates the oocyte and cumulus cells also makes attaching the cumulus cells to the surface easy. In addition to this function, it allows the communication of oocyte and cumulus cells via gap junctions which are mainly comprised of connexin 37 (Gilula et al., 1978).

As granulosa cells produce estrogen, the estrogen level is increasing and eventually reaches its peak level. This level of estrogen causes GnRH secretion to the anterior pituitary, resulting in a sudden LH surge. As a result of this LH surge, proteolytic enzyme secretion and collagenase activity increase, and collagen fibers which surround the follicle are digested; local contraction of muscles causes the ovarian wall to weaken. Eventually, a mature follicle can pass through this wall and ovulation occurs (Richards et al., 2015). The ovulation process includes 5 steps which are the expansion of the cumulus-oocyte complex, breaking down of gap junctions, the continuation of cellular division in the oocyte, releasing of the COC and luteinization (Richards, 2018).

1.6 Luteinization and Corpus Luteum Formation

The term luteinization can be defined as developing corpus luteum (CL) from postovulatory follicular cells as a result of LH surge. Luteinization is an important process for embryo implantation and survival of early embryos since CL formation is essential for progesterone synthesis (Spencer & Bazer, 2002). The corpus luteum can be defined as an endocrine organ composed mainly of theca cells and granulosa cells; even though it is a transient organ, its formation is crucial. Not only LH surge but also differentiation of remaining follicular cells and neovascularization contribute to the formation of CL. The most important responsibility of CL is progesterone synthesis to prepare the endometrium for a possible pregnancy (Terranova, 1986). Until the placenta takes over the duty of progesterone synthesis, CL continues to synthesize progesterone if fertilization occurs. In the case of no fertilization, CL eventually loses its function and regresses (Shrestha et al., 2019a).

Luteinization is a process of morphological, intracellular, and expressional changes in follicular cells which triggers terminal differentiation of these cells by causing them to exit the cell cycle (Murphy, 2000). One of these morphological changes that occur during luteinization is the increasing volume of granulosa cells which results in the growth of CL (M. F. Smith et al., 1994). At ovulation, the theca and granulosa layers are separately luteinized and at the end of the luteinization process, luteinized theca cells represent a small population of CL structure since most of it is composed of luteinized granulosa cells (Shrestha et al., 2019b). In addition to these, in vitro luteinization is associated with intracellular lipid droplet accumulation in granulosa and theca cells which contribute to cholesterol storage for steroidogenesis (Talbott et al., 2020). These morphological alterations cause the follicular cells to change their main product from other steroids to progesterone in *in vitro* conditions and this is the most important functional change after luteinization. Not only progesterone production but also, expression of steroidogenic enzymes such as StAR, CYP11-A, 3β-HSD is increased after luteinization. However, the expression of the enzymes responsible for androgen and estrogen synthesis is highly reduced concerning CL development (Murphy, 2000). CL is composed of multiple cells including steroidogenic luteal cells having theca or granulosa origin and nonsteroidogenic endothelial cells, some immune cells, pericytes, etc. in which luteal granulosa cells are responsible for progesterone synthesis (Fraser & Wulff, 2003; Reynolds et al., 2000). In mammals, the amount of progesterone synthesized by CL is nearly three times higher than the steroid production of follicular cells which indicates that the used cholesterol amount is much higher in CL (Chin et al., 2004).

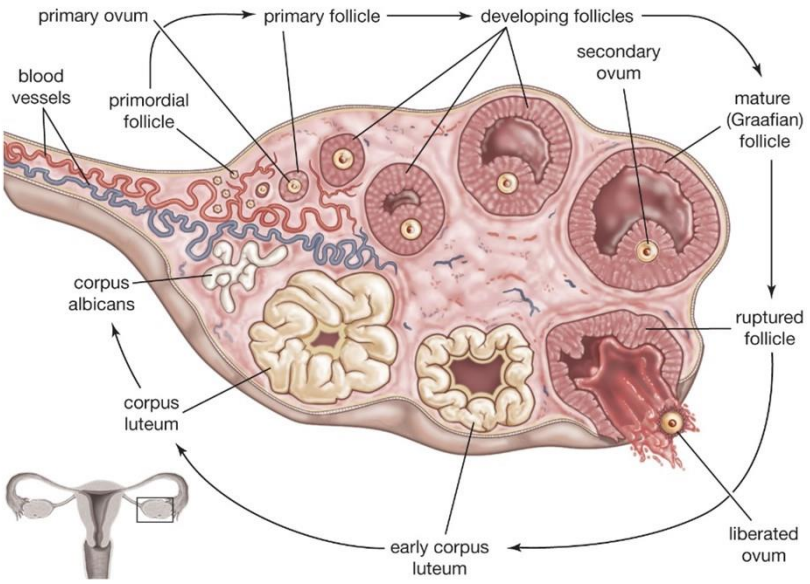

Figure 2: Ovulation process and corpus luteum formation (Hennebold, 2018)

During the formation of the corpus luteum, there are many LH-mediated events to be completed which are tissue remodeling, functional and morphological differentiation of theca and granulosa cells, muscle contraction and angiogenesis (Duffy et al., 2019). The angiogenic factors VEGFs, which are triggered by LH surge, are responsible for the vascularization process during corpus luteum formation (Reynolds et al., 2000). The process of corpus luteum formation has many similarities with inflammatory processes since many of the genes involved in this process are also associated with inflammatory responses (Robker et al., 2000).

1.6.1 Regulation of luteinization

Regulation of luteinization is achieved by activation of some intracellular pathways such as protein kinase A (PKA), protein kinase C (PKC), tyrosine kinase-mediated pathways and phosphatidyl-inositol 3-kinase (PI3K) in the preovulatory follicles (Duffy et al., 2019).

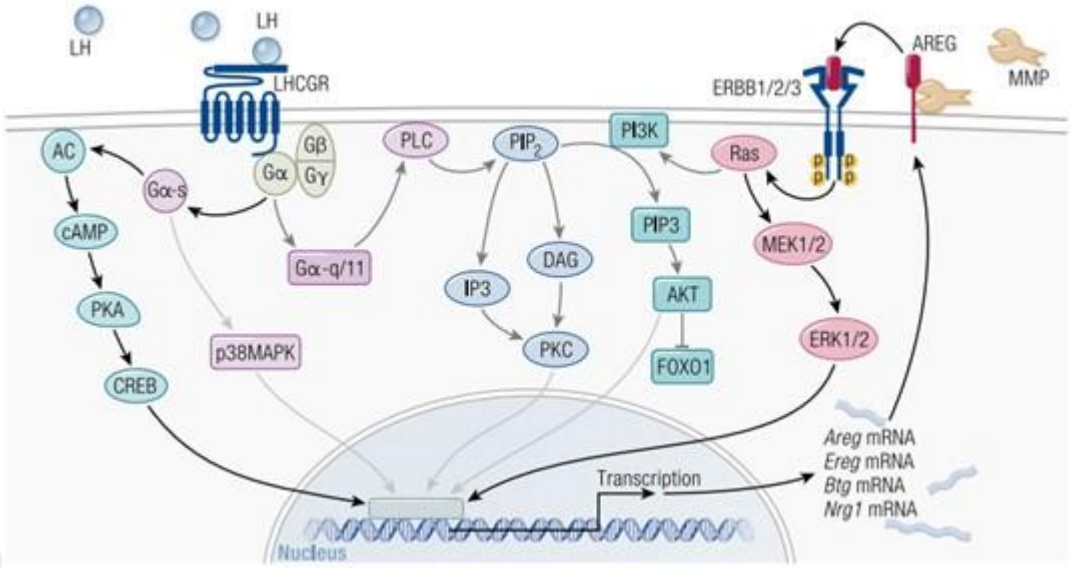

Figure 3: Signaling pathways activated after LH surge in the ovulatory follicle (Duffy et al., 2019)

When LH binds to its receptors on the cell membrane, it activates the adenylate cyclase/ cyclic AMP (cAMP)/ protein kinase A (PKA) pathway (Chin et al., 2004). As a result of this activation, intracellular cAMP level increases and activates cAMP-dependent PKA which further leads to the activation of cAMP response element-binding protein (CREB) (Panigone et al., 2008). It is the primary pathway that regulates the LH/hCG action of the ovulating follicle. In addition to this pathway, phospholipase C (PLC)-PKC and PI3K pathways are also activated concerning the LH surge (Woods & Johnson, 2007; Breen et al., 2013). Moreover, previous findings showed that phosphorylation of p38MAPK increases with LH surge (Maizels et al., 2001), and sudden activation of epidermal growth factor receptor (EGFR)-tyrosine kinase pathway is caused by cAMP-PKA pathway activation by LH surge and an increase in expression of EGF-like factors AREG, EREG, NRG1 and BTG (Duffy et al., 2019).

For regulation of luteinization, oocyte also has two important roles which are maintaining the follicular phenotype by secreting estrogen while preventing the progesterone synthesis (Vanderhyden & Tonary, 1995) and suppressing the LH receptor expression on the granulosa cell surface (Eppig et al., 1997).

1.6.2 Terminal differentiation of granulosa cells

After LH surge, granulosa cells have undergone a process called terminal differentiation by losing their mitotic activity with an arrest in the G1 phase of the mitotic division (Hampl et al., 2000). The dephosphorylation of the retinoblastoma proteins p107 and p130 which are known as mitotic initiators and binding of CDK inhibitors to the cell cycle promoter cyclins cause the terminal differentiation of mitotic activity of the granulosa cells (Robker & Richards, 1998). The most common CDK inhibitors found in the granulosa cells are $p27^{Kip1}$ and $p21^{Cip1}$ (Murphy, 2004).

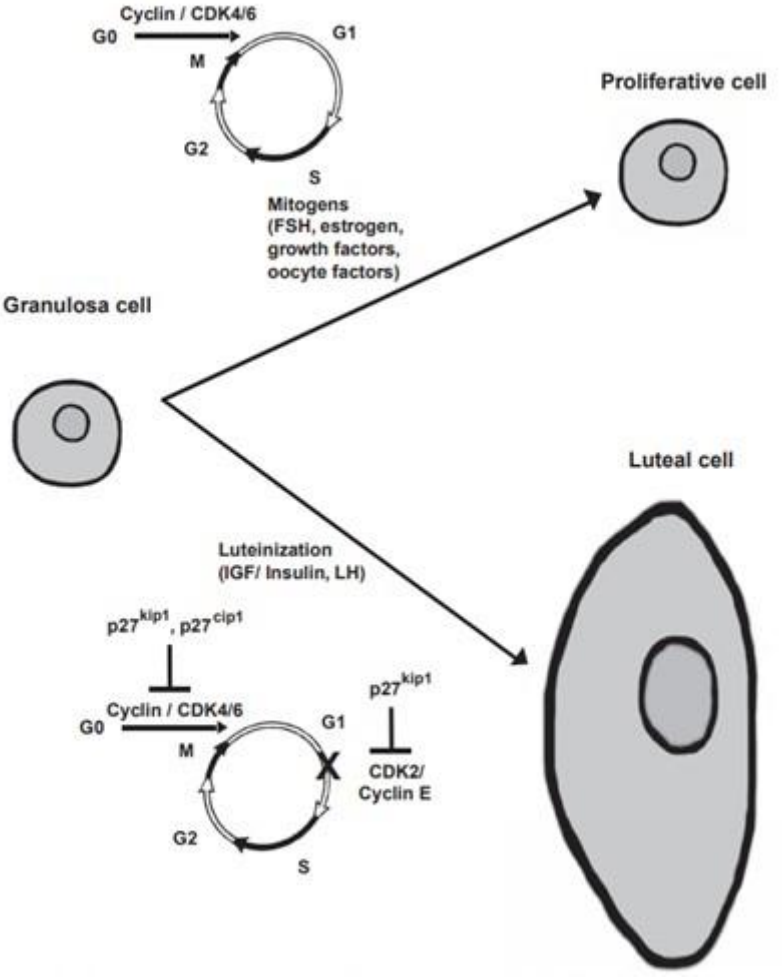

Figure 4: The decision of proliferation or luteinization in granulosa cells (Murphy, 2004)

1.7 Steroidogenesis

The term steroidogenesis refers to a process of active steroid hormone production from cholesterol in which multiple enzymes have a role (Andersen & Ezcurra, 2014). In mammals, the main steroid-producing organs are adrenal glands and gonads especially testicular Leydig cells, placental syncytiotrophoblasts, granulosa, and theca cells of the ovary (Miller & Auchus, 2011). Steroid hormones are categorized into 3 groups; adrenal glucocorticoids, mineralocorticoids, gonadal androgen, and estrogen which have a role in maintaining homeostasis through glucose metabolism, sex differentiation, immunity, reproduction, etc. (Miller, 2013). There are different sources of cholesterol to use during steroid hormone synthesis such as *de novo* synthesis, intracellular store of cholesterol such as stored in lipid droplets as sterol esters, and cholesterol esters taken by endocytosis with lipoprotein receptors (Connelly, 2009). Intracellular de novo synthesis of cholesterol which is controlled by sterol regulatory element-binding proteins (SREBPs) depends on the activity of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) enzyme which is the rate-limiting enzyme in cholesterol biosynthesis (Gwynne & Iii, 2014). This way of producing cholesterol is not enough for that much progesterone synthesis by CL; therefore, endocytosis of cholesterol by low-density lipoprotein (LDL) and high-density lipoprotein (HDL) receptors is the primary source for steroidogenesis in CL. Cholesterol is found in the cell in two forms which are known as free cholesterol and cholesterol esters (Maxfield & Menon, 2006). Free cholesterol is the main substrate for steroid hormone synthesis, while cholesterol esters are the esterified form of cholesterol by 3β hydroxyl group to polyunsaturated fatty acids which are collected in the rough endoplasmic reticulum and eventually give rise to cytoplasmic lipid droplets (Christenson & Devoto, 2003).

1.7.1 Cholesterol acquisition and trafficking

There are two mechanisms for cholesterol transportation within the cell, vesicular or nonvesicular transportation. Cholesterol found in membranes forms lipid or protein rafts to facilitate protein trafficking in the cell (Harayama & Riezman, 2018). It is referred to as “nonvesicular” when cholesterol binds to proteins for transportation; by contrast, if a budding occurs to form a vesicle for transportation from a membrane, and cholesterol is

transported via vesicular cargo, this is called “vesicular” transportation (Maxfield & Tabas, 2005).

The plasma membrane of the cell is known to have the highest amount of free cholesterol originating from plasma lipoproteins and the synthesis of de novo sterol (Miller, 2017a). These sterols are not stable in the plasma membrane; instead, they cycle through the cell regularly and turn back to the membrane. While cycling through the cell, some of these sterols can be metabolized for steroid hormone production, esterification or stored in lipid droplets for further use (Harayama & Riezman, 2018). Through the LDL receptors on the microvilli surface of the membrane, lipoproteins are endocytosed and arrived at lysosomes where they are degraded to lipoprotein cholesterol esters (Maxfield & Menon, 2006). Hydrolyzation of these esters to the free cholesterol is mediated by the acid lipase (LIPA) enzyme. The deficiency of this enzyme is a disorder known as severe acid lipase deficiency or Wolman’s disease which is associated with deposition of cholesterol esters in lysosomes and deficiency in hormone production of steroidogenic cells (Get, 2021). Releasing of free cholesterol from lysosomes is mediated by lysosomal proteins which are NPC1 and NPC2. If the genes that encode these proteins are mutated, a cholesterol storage disorder called Niemann-Pick Type C disease occurs (Strauss et al., 2002). There are also some other sterol binding proteins such as metastatic lymph node 64 protein (MLN64) that have a role in this process (Rigotti et al., 2010). As the steroidogenic cells are stimulated by gonadotropins, an increase in LDL receptors on the cell membrane and internalization of LDL is observed (Harayama & Riezman, 2018). In addition to LDL, HDL is also known to provide cholesterol for steroid hormone production, and its receptor is known as scavenger receptor type B 1 (SR-B1) (Trapani et al., 2012). As in the LDL receptor expression, the number of these receptors also increases with respect to the gonadotropin stimulation. After HDL cholesterol esters are internalized, they are cleaved by neutral cholesterol ester hydrolase (NCEH1) or hormone-stimulated lipase (LIPE) (Strauss, 2019).

Cytoplasmic lipid droplets are another source of cholesterol for steroidogenic cells where almost 80% of esterified cholesterol is found (Gao & Goodman, 2015). Perilipins are found in the membranes of the lipid droplets which mediate the interactions between the lipid droplets and cellular organelles, and help to attach the lipases to the surface of the lipid droplets (Sztalryd & Brasaemle, 2017). Following the stimulation of steroidogenic cells by gonadotropins, protein kinase A phosphorylates perilipins by

cAMP-mediated activity which causes lipases to gain access to sterol esters by the detachment of perilipins from the surface of the lipid droplets (Brasaemle, 2007).

1.7.2 Human steroidogenic pathway and proteins

In figure 5, it is shown that in major human steroidogenesis there are several key enzymes and cofactors. Steroidogenesis begins with the transportation of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane by the activity of StAR protein where it is converted to pregnenolone by the P450 side-chain cleavage enzyme (Andersen & Ezcurra, 2014). Pregnenolone diffuses into the smooth endoplasmic reticulum, and it is converted to progesterone by 3β-hydroxysteroid dehydrogenase (HSD3B) (Chapman et al., 2005).

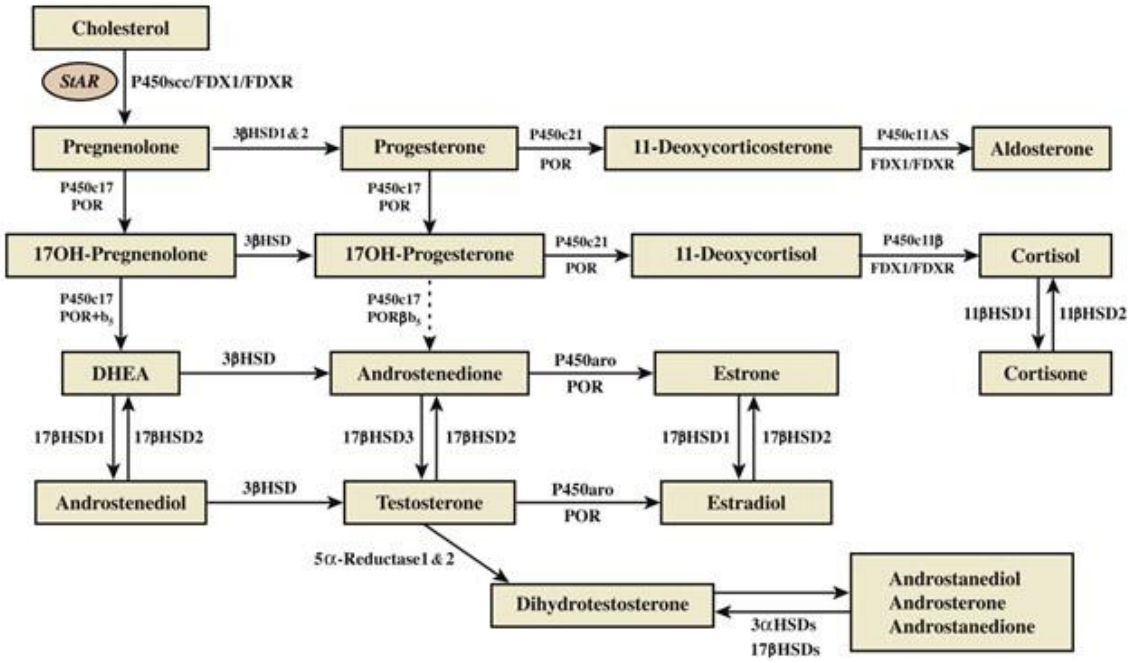

Figure 5: Major steroidogenic pathway in humans (Auchus & Miller, 2015)

1.7.3 Steroidogenesis in the ovulatory follicle

A huge accumulation of lipid droplets in the granulosa cells is seen to provide cholesterol supply for steroid hormone synthesis in response to the midcycle LH surge (Gao & Goodman, 2015). In addition to cholesterol-rich lipid droplet accumulation, the

expressions of the enzymes having important roles in steroidogenesis such as CYP11A1, 3β-HSD, StAR are increased (King & Lavoie, 2012). Since the expression of CYP17A1 is lower with respect to the theca cells, there is a limited conversion of progesterone to the estrogens and androgens in granulosa cells which makes progesterone the major product of the granulosa cell steroidogenesis after ovulation (Xu et al., 2011).

According to the two cell-two gonadotropin theory, granulosa cells are dependent on theca-derived androgens for estrogen synthesis; however, after ovulation, a decrease in the expression of CYP19A1 which results in a decrease in estrogen synthesis occurs (Raju et al., 2013). The major steroid hormone in the follicular fluid changes from estrogen to progesterone after luteinization. There is also the synthesis of other steroids such as cortisol in granulosa cells (Lewicka et al., 2003). Even though the role of cortisol is not known in the ovulation process, it is known that this hormone is an anti-inflammatory hormone and might have a role in the protection of the ovary from the damage caused by ovulation (Hillier & Tetsuka, 1998).

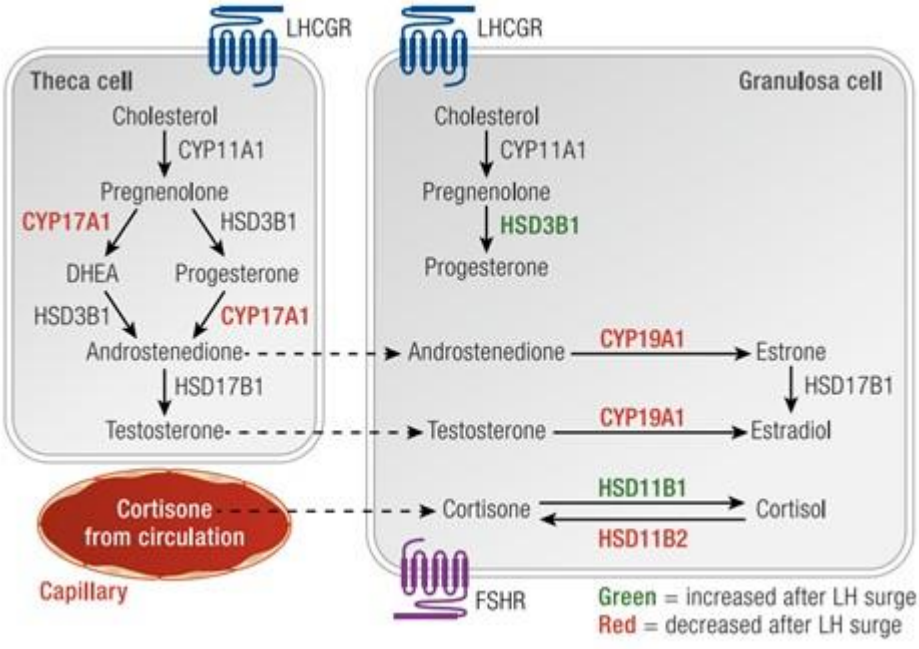

Figure 6: Steroidogenesis in theca and granulosa cells (Duffy et al., 2019)

1.8 Transforming Growth Factor β (TGF-β) Superfamily

TGF-β superfamily members are growth factors that control fundamental cellular processes such as differentiation, proliferation, cellular communication, apoptosis (Lutz & Knaus, 2002). Activin/inhibin family, bone morphogenetic proteins (BMPs), TGF- βs, growth differentiation factors (GDFs), anti-Müllerian hormone (AMH), glial cell-derived neurotrophic factors (GDNFs), and their subfamilies are the examples of TGF- β superfamily members and have crucial roles in follicular development, maturation of the oocyte, implantation process, etc. (Knight & Glister, 2006) and their mutations or deletions can result in some disorders in the reproductive system (Qin et al., 2015). Except for inhibin and GDNF subfamily members, TGF- β superfamily members bind to Serine/Threonine kinase receptors, type I and type II receptors (Massague, 2000). First of all, ligands of TGF-β superfamily members bind to type II receptors and form a complex which causes recruitment and activation of type I receptor and this causes phosphorylation of receptor-regulated SMADs (R-SMADs) and activates them (Shi & Massagué, 2003; Massague, 2000). In mammalians, there are seven type I receptors which are known as activin receptor-like kinases (ALKs), and five type II receptors which are TβR2, AMHR2, BMPR2, ACVR2A, and ACVR2B (Massagu, 1998). Their localization in the ovary is shown in table 2.

Table 2. Localization of TGF-β receptors in the ovary

Receptors	**Localization in the ovary**
ALK2	Luteal granulosa cells
ALK3	Luteal granulosa cells, oocyte, corpus luteum, granulosa cells
ALK4	Stroma or fetal ovary, pre-granulosa cells
ALK5	Luteal granulosa cells, granulosa cells, theca cells
ALK6	Luteal granulosa cells, granulosa cells, oocyte, corpus luteum, stroma cells
TβR2	Granulosa cells, theca cells
TGFβR3	Granulosa cells of preantral follicles
AMHR2	Granulosa cells of preantral follicles

BMPR2	Granulosa cells, luteal granulosa cells, oocyte, corpus luteum, stroma cells
ACVR2A	Granulosa and theca cells of small antral follicles, secondary follicle's oocyte
ACVR2B	Granulosa and theca cells of small antral follicles

In humans, TGF-β superfamily members play critical roles in the regulation of gonadal development. Starting from induction of primordial germ cells, they have roles in the regulation of preantral follicle development, granulosa cell proliferation, extracellular matrix formation, cumulus-oophorus complex formation, luteal function, ovulation, and corpus luteum formation (Childs et al., 2010; Shi & Massagué, 2003; Drummond, 2005; Knight & Glister, 2006).

1.8.1 Signal transduction pathways of TGF- β

As mentioned before, when a ligand binds to a type II receptor and forms a complex, this complex binds to the type I receptor and activates it. Activation of type I receptor causes phosphorylation of signal transducers of R-SMAD which are the transcription factors SMAD 1-8 (Massague, 2000). Different members of TGF-β activate different SMADs; however, there are two general models of activating R-SMADs. In the first model, via the activation of ALK2, 3, and 6, SMAD1/5/8 responds to AMH, BMPs, and GDFs; while in the second model, via ALK4, 5, and 7, SMAD2/3 respond to TGF βs, activins, and GDFs (Drummond, 2005). After R-SMAD is activated, it associates with common SMAD, also known as co-SMAD or SMAD4, and makes a complex which involves in modulating gene expression by translocating into the nucleus (Itoh et al., 2000; Shi & Massagué, 2003).

Table 3. Localization of TGF-β ligands in the human ovary

Ligand	**Localization in the ovary**
BMP2	Corpus luteum, follicular fluid, luteal granulosa cells
BMP4	Oocyte, follicular fluid, luteal granulosa cells, theca cells

BMP5	Luteal granulosa cells
BMP6	Corpus luteum, luteal granulosa cells
BMP7	Luteal granulosa cells, oocyte, theca cells, follicular fluid
BMP8A	Luteal granulosa cells
BMP15	Preantral follicles, oocyte, cumulus cells, theca cells, granulosa cells, follicular fluid
GDF8	Luteal granulosa cells, follicular fluid
GDF9	Cumulus cells, preantral follicles, luteal granulosa cells, oocyte, follicular fluid
AMH	Granulosa cells, follicular fluids of preovulatory follicles
TGF-β1	Luteal cells of corpus luteum, follicular fluid, granulosa cells
TGF-β2	Small luteal cells of corpus luteum, theca cells
Inhibin α	Granulosa and theca cells of the preovulatory follicle
Inhibin βA	Granulosa and theca cells of antral follicles
Inhibin βB	Granulosa and theca cells of antral follicles
Activin A	Follicular fluid
Inhibin A	Follicular fluid
Inhibin B	Follicular fluid

There are other signaling pathways having a role in mediating TGF-β ligands called SMAD independent pathways. The major pathway activated by TGF- β is the mitogen-activated protein kinase (MAPK) signaling pathway since the studies involve SMAD4 deficient or dominant-negative SMAD cells show that the activation of MAPK in response to TGF-β occurs (Derynck & Zhang, 2003). In addition to MAPK, extracellular-signal-regulated kinase (ERK), p38MAPK, c-Jun N-terminal kinase (JNK), phosphatidylinositol-3-kinase (PI3K), protein kinase A (PKA) signaling pathways are also activated by BMPs and TGF-βs (Funaba et al., 2002; Engel et al., 1999). Moreover, in two studies it is shown that TGF- β1 and GDF8 cause a decrease in StAR expression by ERK and SMAD3 pathways in human granulosa cells (Fang et al., 2015; Fang et al., 2014).

1.9 Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMPs) are the largest subfamily of the TGF- β superfamily and are known as multifunctional growth factors. They have roles in embryonic development especially heart, cartilage, neural development, and postnatal bone formation (Chen et al., 2004). In addition to these organs, they also have roles in different biological events in the ovary such as follicular recruitment, ovulation, steroidogenesis, cellular differentiation, proliferation, and apoptosis (Khalaf et al., 2013; Regan et al., 2018). They have some specific receptors which belong to the serine/threonine kinase receptor family, and they have type I and II subtypes. Type I receptors of BMP2 and BMP4 are ALK3 (BMPRIA) and ALK6 (BMPRIB), type II receptor is BMPRII. Type I receptors of BMP6 and BMP7 are ALK2 (ACVRIA) and ALK6 (BMPRIB), type II receptors are BMPRII, ACVRII, ACVRIIB. For BMP15, the type I receptor is ALK6, and the type II receptor is BMPRII (Shimasaki et al., 2004). The downstream molecules of BMP receptors are SMAD 1,5 and 8 and they regulate the BMP signal transduction (Shimasaki et al., 1999).

When a type of BMP binds to its receptor on the cell surface, the type I receptor is transphoshorylated by the type II receptor. This type I receptor further acts on intracellular signaling SMAD proteins and transphoshorylates them. The type of SMAD which is phosphorylated by type I receptor is called receptor-regulated SMAD or R-SMADs which are mainly composed of SMAD 1,5,8,2,3 (Lagna et al., 1996). The activated R-SMAD interacts with its common partner SMAD4 or co-SMAD and forms a SMAD complex which regulates the expression of the genes targeted by being translocated into the nucleus and interacting with the transcription factors specific to the target gene (Shimasaki et al., 2004).

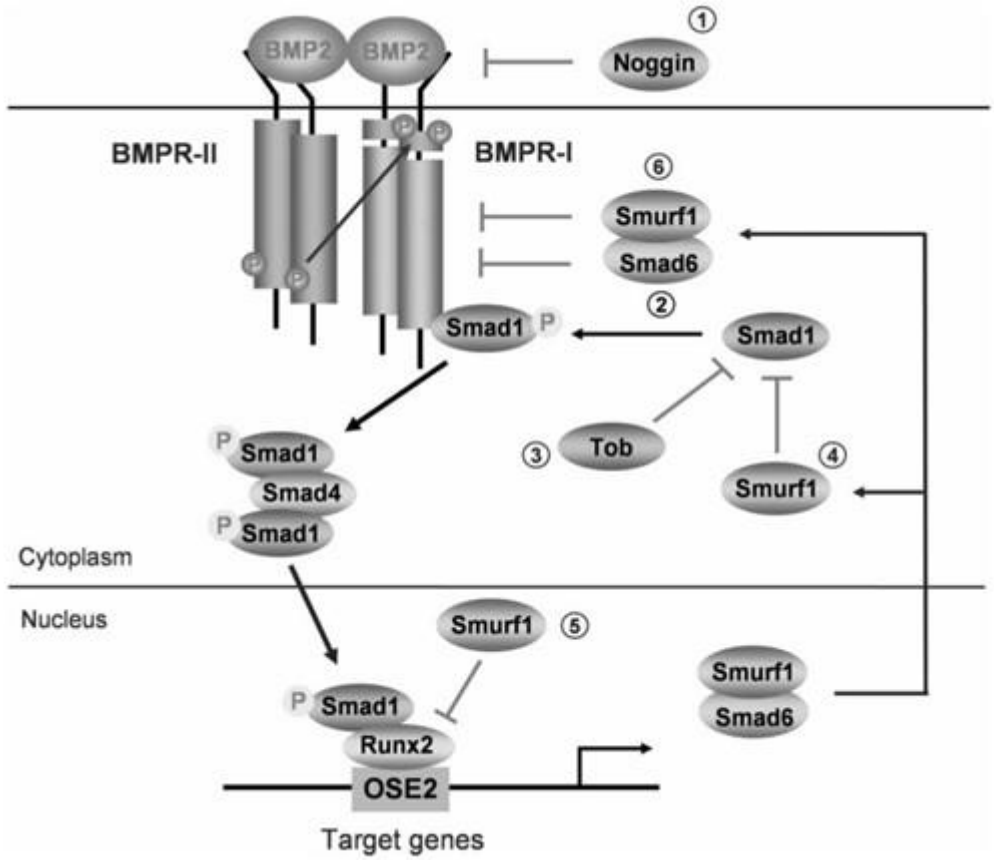

Figure 7: Regulation of BMP signaling (Chen et al., 2004).

As it can be seen in figure 7, regulation of BMP signaling is achieved at different molecular levels. The first one is blocking BMP signaling by binding BMP antagonists such as noggin to BMP2, 4, and 7. It is stated that osteoporosis in mice is associated with the overexpression of noggin in osteoblasts (Wu et al., 2003). Secondly, the binding of SMAD 6 to the type I receptor prevents activation of SMAD 1, 5, and 8. Thirdly, the interaction of Tob with SMAD proteins which are activated by BMPs causes inhibition of BMP signaling. It is shown that bone formation and BMP signaling is increased in Tob null mutant mice (Cao & Chen, 2005). Fourthly, the degradation of SMAD proteins is achieved by Smurf1 which is an E3 ubiquitin ligase, and its interaction with SMAD 1 & 5 causes degradation. In addition to these features, it also mediates degradation of Runx2 which is a bone-specific transcription factor (Zhao et al., 2004). By forming a complex with SMAD 6, Smurf1 leaves the nucleus and targets the type I receptors by causing their degradation (Scherl et al., 2002).

1.9.1 BMP7 and its signaling pathways

BMP7 is a growth factor which belongs to the TGF- β superfamily as indicated in the previous section. Several tissues such as the heart, brain, liver, pancreas, bone marrow, lymph nodes, skeletal muscle express BMP7 (Narasimhulu & Singla, 2020), and it has important roles in different biological processes like development of the nervous system, eye and kidney formation, regeneration and repair of the skeletal system, bone homeostasis and calcium regulation (Dudley et al., 1995). In the reproductive system, BMP7 is expressed by the testis, ovary, placenta, and uterus. It has important roles in embryogenesis, the transition of primordial follicles to primary follicles, preantral and antral follicle growth from primary follicles (Oktem & Urman, 2010).

BMP7 is produced by the cells as 431 aminoacids in the form of pro-protein which includes signal peptide (29 aminoacids), leader peptide (263 aminoacids), and mature peptide (139 aminoacids). A furin-like proteinase hydrolyzes pro-BMP7 and converts it to mature-BMP7 during processing (Bustos-Valenzuela et al., 2010). BMP7 can form both homodimers, and heterodimers with BMP2 and BMP4. It has been stated that the biological activity of BMP7 is higher when it is in heterodimer form with other BMPs (Little & Mullins, 2009).

When BMP7 binds to its receptor on the cell surface, it activates different cellular pathways which can be classified as SMAD-dependent (canonical) pathway and SMAD-independent (non-canonical pathway) (Boon et al., 2011). As shown in figure 8, in the canonical pathway when BMP7 binds to BMPR2 on the cell surface, it causes phosphorylation and activation of SMAD 1/5/8 in the cytoplasm. Further, these phosphorylated regulatory SMADs form a complex with SMAD 4 and transduce into the nucleus to activate cofactors and transcriptional factors to regulate gene expression (Miyazono et al., 2010). In the non-canonical pathway, activation of the receptor by binding BMP7 initiates the MAPK signaling by the interaction of several proteins. First, X-linked inhibitors of apoptosis protein (XlAP) and its downstream molecules TGF-β activated kinase 1 (TAK1) and TAK1 binding protein (TAB1) are activated which further activate NFkB, p38, JNK, and ERK pathways in their downstream (Yamaguchi et al., 1999). These pathways are the major pathways controlling cellular differentiation, cell death, and cellular migration. There are several studies conducted to understand the mechanism of BMP7 via signaling through these pathways, and it has stated that cell

proliferation which is regulated by the p38 MAPK pathway increased with a low dose of BMP7, while cell proliferation which is regulated by the SMAD pathway suppressed with a high dose of BMP7 (Hu et al., 2004). In addition to these pathways, by either SMAD dependent or binding of BMP7 to its receptor, PI3K is activated and this causes phosphorylation of phosphatidylinositol biphosphate (PIP2) to phosphatidylinositol triphosphate (PIP3) (Shimizu et al., 2012) which leads to AKT phosphorylation and activation of mTOR pathway.

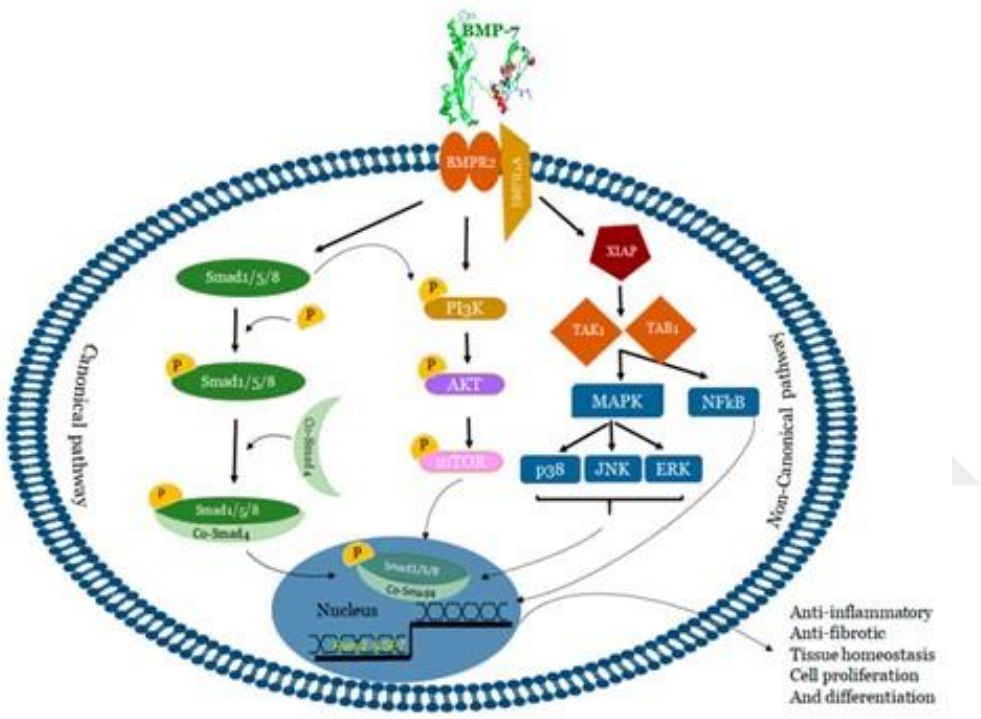

Figure 8: Signaling pathways of BMP7 (Narasimhulu & Singla, 2020)

There are some important antagonists of BMP7 which bind to the receptors and control signaling mechanisms of BMP7 in the cell. The most important antagonists are noggin, follistatin, and chordin for BMP7. By blocking the effects of BMP7 in the differentiation of osteoblasts, noggin inhibits limb development (Gazzerro et al., 1998). Follistatin prevents SMAD1/5/8 pathway activation by inhibiting the binding of BMP7 to its receptor BMPR2 (Amthor et al., 2002). Similarly, by inhibiting the binding of BMP7 to its receptor, chordin prevents phosphorylation of downstream pathways and leads to deficiencies in several biological actions (Larman et al., 2009).

It was shown by Shimasaki *et al.* in 2004 that theca cells of secondary follicles produce BMP7 (Shimasaki et al., 2004) and Abir *et al*. showed that human fetal follicles express BMP7 mRNA in granulosa cells and oogonia (Abir et al., 2008). In 2004, Lee *et al*. demonstrated that stimulation of FSH receptors and growth of primordial follicles depend on BMP7 in murine ovaries (Woo Sik Lee et al., 2004). In addition to these studies, Shi *et al*. stated that LH receptor gene expression is decreased with BMP7 treatment in human granulosa cells (J. Shi et al., 2010). Miyoshi *et al*. showed that aromatase expression increases with BMP7 which favors the estradiol production in rat granulosa cells in vitro (Miyoshi et al., 2007). During ovulation progesterone has an important role, and it was stated by Lee *et al*. in 2001 that BMP7 inhibits progesterone production which might be related to inhibition of ovulation in the rat (W. S. Lee et al., 2001). However, the effects of BMP7 on human steroidogenic function and hormone production of granulosa cells after luteinization is poorly understood. Therefore, it is aimed to address the effects of BMP7 on steroidogenesis in human luteal granulosa cells in this thesis.

Chapter 2: **MATERIALS & METHODS**

2.1 Study Design, Size and Duration

It is an in vitro translational research study on corpus luteum biology conducted with human luteal granulosa cells collected in 2021 at Koç University Hospital and Amerikan Hospital.

2.2 Patients

Human luteal granulosa cells used in this study were obtained from follicular aspirates of 10 normo-responder patients undergoing ovarian stimulation with recombinant FSH and GnRH antagonist protocol during assisted reproduction treatment.

2.3 Chemicals and Reagents

The recombinant form of hCG (Ovitrelle) was purchased from Merck Global (Darmstadt, Germany), recombinant human activin-A was purchased from Cell Guidance Systems, recombinant human BMP7 was purchased from R&D systems (#354-BP-010). The antibodies 3β-HSD Type II (sc-100466) and StAR (sc-166821) were purchased from Santa Cruz Biotechnology, and aromatase (ab34193) monoclonal mouse antibody was purchased from Abcam Inc. Anti-Vinculin antibody was purchased from Sigma-Aldrich (Germany). DMEM: F12 culture medium was purchased from Lonza (#BE12-719F), fetal bovine serum (S181H-500), and PenStrep (L0018-100) were purchased from Biowest and Amphotericin B was purchased from Sigma (1397-89-3). Radioimmunoprecipitation assay (RIPA) buffer was purchased from Sigma-Aldrich (R0278). Other chemicals and reagents for western blotting were purchased from BioRad. Light Cycler 480 SYBR Green I Master was purchased from Roche (#04707516001).

2.4 Isolation and Culture of Human Luteal Granulosa Cells

Luteal GCs were isolated from follicular aspirates collected during the oocyte retrieval procedure.

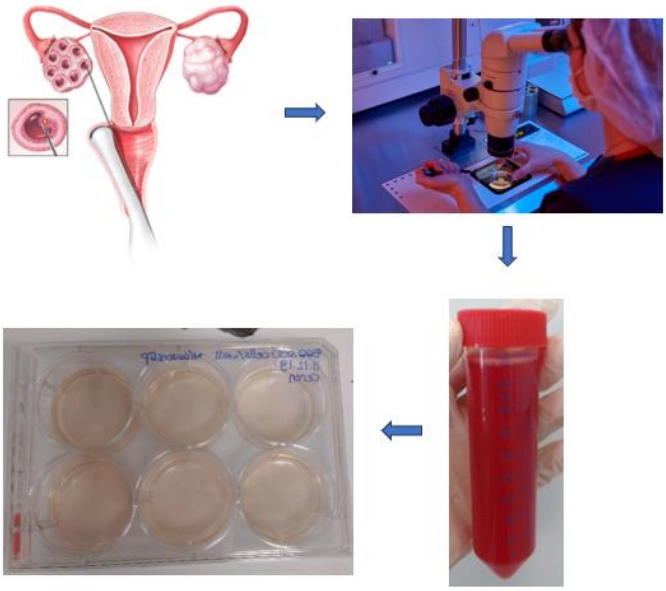

Figure 9: Isolation and culture of human luteal granulosa cells

These follicular fluids taken from each patient were collected in 50 ml centrifuge tubes and centrifuged at room temperature for 5 minutes at 500 x g. After centrifugation, the supernatant was aspirated with the help of a glass Pasteur pipette which was connected to a vacuum line in a level 2 biological safety cabinet. At this step, no layer formation was seen in the pellet, and it was important to eliminate the risk of erythrocyte contamination. In order to do that hypo-osmotic lysis technique was used (Lobb & Younglai, 2006).

After supernatant aspiration, filtered double distilled water (9 ml) was added to the cell pellet remaining in the bottom of the tube and mixed. After approximately 20- 30 seconds, 1 ml of 10X concentrated PBS (Gibco, # 70011044), pH 7.4, was added and mixed. The tubes were centrifuged at 500 × *g* for 5 minutes at room temperature. The cell pellet was re-suspended in 3 ml of DMEM: F12 culture medium (Lonza, #BE12-719F) supplemented with 10% fetal bovine serum (Biowest, S181H-500), 1% PenStrep (Biowest, L0018-100), and 1:1000 Amphotericin B (Sigma, 1397-89-3); then counted for cell number and viability in 0.2% trypan blue (Gibco, #15250061) on a hemocytometer.

The recovered cells were either cultured or processed for other experiments. For western blotting, cells were seeded in six-well format culture plates at a density of 500.000 cells per well; for immunofluorescence staining, seeded in 24-well format culture plates on 12 mm coverslips at a density of 100.000 cells per well using culture media. The cells were incubated at 37°C and 5% CO_2 for at least 24 hours to adhere to the culture plate. The next day, their viability, adherence, and confluency were checked under the microscope. If there was no contamination and at least 80% of the cells were attached to the culture plate in clusters, the cells were ready for treatment.

2.5 Treatments for primary cell culture

For treatments of the cells hCG, activin-A and BMP7 were used, respectively. The recombinant form of hCG (Ovitrelle) was used at 10 IU concentrations. Recombinant human activin-A was used at 25 ng/ml concentration. Recombinant human BMP7 arrived in lyophilized form as 10 µg. It was reconstituted as indicated in the datasheet at 100 µg/mL in sterile-filtered 4 mM HCl which contains 0.1% human serum albumin (Sigma, SRP6182-1MG) and it was used at 50 ng/ml concentration.

10 IU hCG, 25 ng/ml activin-A, and 50 ng/ml BMP7 were mixed with the culture media, and cells were incubated with these media for 24 hours at 37°C and 5% CO_2.

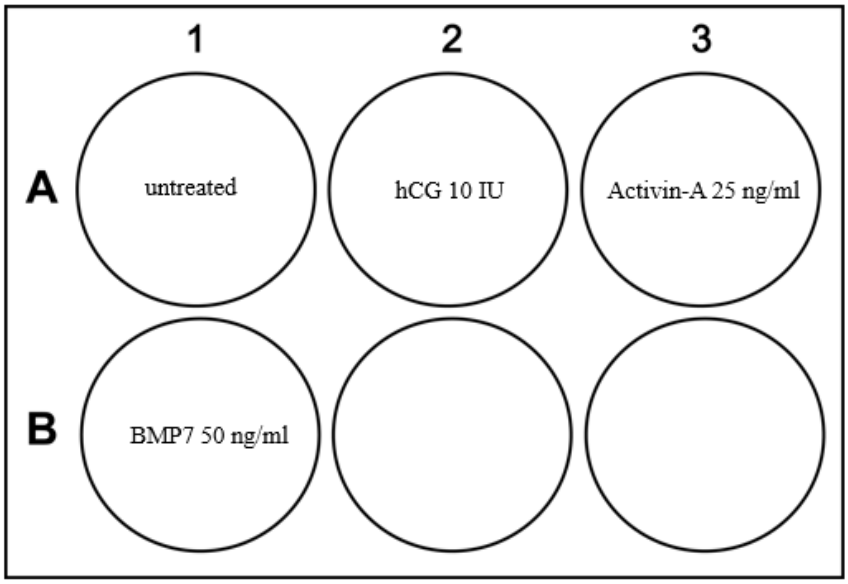

Figure 10: Treatment set-up for HLGCs. hCG, Activin-A, and BMP7 treatments with optimum concentrations.

2.6 Hormone Assays

24 hours after treatment, 1 ml of culture media was collected and kept for hormone analysis at -20°C. Estradiol (E_2) and progesterone (P_4) levels were determined by using electrochemiluminescence immunoassay "ECLIA" via Roche Cobas-6000 analyzer (Roche, Mannheim, Germany). Lower detection limits of E_2 and P_4 were 5.00 pg/ml and 0.05 ng/ml, respectively.

2.7 Western Blotting

2.7.1 Protein Isolation

After storing samples for hormone assays, the culture media were discarded by using a glass Pasteur pipette which is connected to a vacuum line in sterile conditions. The culture plate was washed with 1X DPBS (Gibco, #14190144) once, then the cells were trypsinized by using 0.05% trypsin/EDTA (Biowest, #25300054). After cells were detached from the plate, the activity of trypsin/EDTA was inactivated by adding culture media onto the trypsin-cell suspension. Then, the cells were collected into properly labeled 1.5 ml Eppendorf tubes and centrifuged at 500 x g for 5 minutes. After centrifugation, the supernatant was discarded, and the cell pellet was washed with 1X DPBS once and centrifuged at 500 x g for 5 minutes. Again, the supernatant was discarded, and proteins were extracted from cells by using radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, R0278). After cells were dissolved in the RIPA buffer, eppendorf tubes were vortexed at least 6 times with 5 minutes intervals in 30 minutes. Then, tubes were centrifuged at 14.000 x g for 20 minutes at 4°C. After centrifugation, supernatants were collected to new properly labeled eppendorf tubes on ice. Protein concentrations of the samples were quantified by the BCA protein assay kit (Intron, #21071) according to the instructions of the kit as mentioned below.

2.7.2 BCA Protein Assay

In this kit, there was solution A, solution B, and BSA solution (2 mg/ml), and the kit was stored at room temperature. As the first step of the BCA assay, protein standards were prepared according to the instructions. Working solutions were prepared by mixing 50 parts of solution A and 1 part of solution B. It was calculated according to the total number of standards and samples as 200 µl will be added to each well. 96 well culture plates were used for this assay. Samples were diluted at 1:5 by using nuclease-free water. 10 µl of every standard and sample were added to the wells as replicates. Then, 200 µl of BCA working reagent was added to every well carefully. The plate was covered with aluminum foil and mixed on a plate shaker for 30 seconds. Then, the plate was placed at 37°C nonsterile incubators and waited for 30 minutes. After 30 minutes, the plate was cooled to room temperature and the absorbances were measured at 562 nm. After appropriate calculations, the protein amounts were decided for western blot analysis, and 20 µg of protein was loaded into each well.

Solutions and reagents needed for western blotting: running, transfer, and blocking buffers

Running buffer (Tris-Glycine/SDS)

For 1 L;

25 mM Tris base

190 mM glycine

0.1% SDS

Check the pH and adjust to 8.3.

Transfer buffer (semi-dry)

For 1 L;

25 mM Tris

190 mM glycine

20% methanol

0.1% SDS

Check the pH and adjust to 8.3.

10X Tris-buffered saline

For 1 L;

24.23 g Trizma HCl

80.06 g NaCl

Dissolve in 800 mL distilled water

pH to 7.6 with HCl

Complete the volume to 1 L.

1X TBST

For 1 L;

100 mL TBS 10X

900 mL distilled water

1 mL Tween 20

Blocking buffer

5% nonfat dry milk

Dissolve nonfat dry milk in TBST buffer, mix well.

The required volume of 4X Laemmli sample buffer (Bio-Rad, #161-0747) and β-mercaptoethanol (Bio-Rad, #1610710) (1:10) mixture was added into the samples. In order to denature samples, they were boiled at 95°C for 5 minutes and spun down after boiling.

2.7.3 Gel electrophoresis

Mini-PROTEAN TGX precast gels (Bio-Rad, #4561094) were used for gel electrophoresis. Equal amounts of protein were loaded (20 µg) into the wells along with molecular weight markers; for this purpose, 3 µl of precision plus protein dual-color ladder (Bio-Rad, #1610374) was used. The loaded gel was run at 80 Volt for approximately 1.5 hours.

2.7.4 Semi-dry Transfer

The extra thick blot filter papers and polyvinylidene fluoride membrane are pre-wetted with a transfer buffer. Before placing it in the transfer buffer, the PVDF membrane was activated by soaking it with 100% methanol. After running was completed, the gel was placed in a small container with some running buffer. One of the pre-wetted blot papers was placed onto the platinum anode and with the help of a roller, air bubbles were excluded. Then, a pre-wetted nitrocellulose membrane was placed onto the blotting paper with a proper alignment. At this point, it was important not to let the membrane dry out. After that, the gel was placed onto the membrane carefully and a roller was used to exclude air bubbles. Finally, another blot paper was put onto the gel and air bubbles were again excluded carefully. The cathode plate was placed onto the stack and the latches were engaged by pressing onto it carefully. Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit (#170-4272, BioRad, USA) was used for semi-dry transfer. After the transfer was completed, the power was turned off and layers of this sandwich were peeled carefully to reach the membrane. Then, the membrane was rinsed with tap water and stained with Ponceau solution in order to check the quality of the transfer. After the excess parts of the membrane were cut off, the remaining parts were washed with TBST to get rid of the Ponceau stain. When the membrane was cleaned enough, it was ready for blocking. For blocking, 5% nonfat dry milk was used, and membranes were incubated in nonfat dry milk for 1 hour at room temperature. After 1 hour, membranes were rinsed with TBST 3 times for 5 minutes. Then, they were ready for primary antibody incubation.

2.7.5 Antibody Incubation

Primary antibodies were diluted in 2% BSA solution with NaN_3 according to the instructions of the manufacturer and membranes were incubated overnight at 4°C. On the next day, primary antibodies were collected for further use, and membranes were rinsed with TBST 3 times for 5 minutes. HRP conjugated secondary antibodies were diluted in 5% nonfat dry milk with a 1:2000 ratio. Membranes were incubated with secondary antibody solution for 1 hour at room temperature. After 1 hour, the membranes were rinsed with TBST 3 times for 5 minutes, and they were ready for visualization.

2.7.6 Visualization

For quantification of proteins, Pierce Enhanced Chemiluminescence (ECL) substrate (Thermo Fisher, #32106) was used in a 1:1 ratio. Membranes were incubated with enough amount of ECL for 1 minute and then detection of chemiluminescence was performed with Chemidoc XRS+ Imaging System (BioRad) and ImageLab software (BioRad, #1708299) was used for further applications.

2.8 Gradient PCR

In order to determine the optimal annealing temperature for primers, the gradient PCR method was used. For 6 different temperatures, samples were prepared as with cDNA and without cDNA. The total volume of each tube was 25 µl and in each tube 12.5 µl Dream Taq Green PCR Master Mix (ThermoFisher, #K1081), 2 µl primer mix (F+R), and NFW were mixed. For the tubes containing cDNA, the amount of NFW was 8.5 µl and 2 µl cDNA was added at the end. For the tubes without cDNA, 10.5 µl NFW was used. cDNA concentration in each tube was 25 ng/ml. In total there were 12 PCR tubes for each primer, and they were run at three different stages.

Stage 1: 3 minutes at 95 °C
Stage 2: 30 seconds at 95 °C
30 seconds at different temperature zones } 35 cycles
30 seconds at 72 °C
Stage 3: 10 minutes at 72 °C

After running was completed, the samples were run in 2% agarose gel. 2 gr of agarose (ThermoFisher, #17850) was weighed and dissolved in 100 ml 1X TAE buffer (ThermoFisher, #B49) in a microwave oven for approximately 3-4 minutes. After agarose was dissolved, it was cooled down under the water by shaking (to prevent solidification) and 3 µl of EtBr was added. After that, the gel was poured into a PCR mechanism and left to dry.

After the gel was dried, the mechanism was placed on the running platform filled with a 1X TAE buffer, and combs were removed carefully. A 100 bp gene ruler was loaded to

the first well and the samples were loaded according to their increasing temperature and cDNA presence or absence. The temperatures were 56.0 °C, 58.0 °C, 60.0 °C, 61.0 °C, 63.0 °C, 64.0 °C, respectively. When loading was completed, the running was started with a constant 90 mV. Approximately 30 minutes running was completed, and the gel was visualized by Chemidoc XRS+ Imaging System (BioRad).

2.9 qRT-PCR

2.9.1 RNA isolation

After cells were trypsinized by the same procedure as done in the protein isolation step of western blotting, RNA was isolated by using a quick-RNA micro prep kit (Zymo-Research, #R1050) according to the instructions and RNA concentration was measured by using a Nanodrop spectrophotometer (Thermo Fisher Scientific, #ND-2000).

2.9.2 cDNA synthesis

After RNA was isolated, the appropriate amount of RNA was calculated in order to synthesize 1000 ng cDNA. By using the iScript cDNA synthesis kit (Bio-Rad, #1708890), the reaction mix was prepared from the appropriate amount of RNA calculated before according to the instructions in the kit. After that, all of the complete reaction mixes were incubated in a T100 thermal cycler (Bio-Rad, #1861096). The reaction steps were priming for 5 minutes at 25°C, reverse transcription for 20 minutes at 46°C, RT inactivation for 1 minute at 95°C. After all of the steps were completed, cDNA was stored at 4°C for further use.

2.9.3 qRT-PCR

For detecting and comparing the mRNA expressions of the specific genes by quantitative real-time PCR, Light Cycler 480 SYBR Green I Master (#04707516001, Roche) was used. The list of the primers and 3’ to 5’ sequences were shown below.

Table 4. List of primers

GAPDH	F: ATGGAAATCCCATCACCATCTT
	R: CGCCCCACTTGATTTTGG
ACVR1	F: GACGTGGAGTATGGCACTATCG
	R: CACTCCAACAGTGTAATCTGGCG
BMPR1A	F: CTTTACCACTGAAGAAGCCAGCT
	R: AGAGCTGAGTCCAGGAACCTGT
BMPR1B	F: CTGTGGTCACTTCTGGTTGCCT
	R: TCAATGGAGGCAGTGTAGGGTG
BMPR2	F: AGAGACCCAAGTTCCCAGAAGC
	R: CCTTTCCTCAGCACACTGTGCA

RT-PCR protocol:

The hold stage was completed with a velocity of 2.8°C/s from 25°C to 95°C and it was completed at 95°C for 5 minutes. The PCR stage started at 95°C for 10 seconds, then the temperature decreased to 60°C with a velocity of 2.1°C/s and after 30 seconds, the temperature increased to 72°C with a velocity of 2.1°C/s and continued for 20 seconds. The number of cycles for the PCR stage was 40. After 40 cycles were completed, the melt curve stage started with a temperature increase to 90°C at a velocity of 1.6°C/s. After 5 seconds, it decreased to 65°C with a velocity of 1.6°C/s. After 1 minute, with a velocity of 0.05°C/s, the temperature increased to 97°C for 15 seconds. When this stage was completed, the temperature decreased to 40°C with a velocity of 2°C/s for 10 seconds for the hold stage.

All the samples had three technical replicates in the same assay, and their mean Ct and SD values were calculated and analyzed by the ΔΔCt method to indicate relative quantitation of target gene expressions.

2.10 Immunofluorescence Staining

Phosphate Buffer (0.2 M)

4,15 gr sodium phosphate monobasic

22,5 gr sodium phosphate dibasic

Double distilled water

Add 800 ml dH_2O and adjust pH to 7.4 using 1 N NaOH or 1 N HCl.

Complete to volume 1 L.

Store at room temperature.

4% PFA (1 L)

40 gr paraformaldehyde

500 ml 0.2M phosphate buffer (pH:7.4)

500 ml dH_2O

1 N NaOH

Paraformaldehyde was weighed under a fume hood (because it is toxic) and dissolved in 400 ml dH_2O. With the help of a thermometer, the temperature was measured until 58-60 ^oC and during this time 1 N NaOH was being added to the solution drop by drop until it looks transparent. Then, a phosphate buffer was added to this solution and was waited to cool down. pH was adjusted to 7.4, properly labeled, and stored at 4 ^oC.

2.10.1 Fixation

After discarding used media, wells were washed with 1X DPBS once. In order to fix the cells, sterile 4% paraformaldehyde was used. The surface of the coverslips was covered with the appropriate amount of 4%PFA (400-500 µl) in sterile conditions and waited for 20 minutes at room temperature. Working under sterile conditions was not important after this step. After 20 minutes, PFA was aspirated by a vacuum line and coverslips were washed with 1X DPBS two times. Then, the wells which involve coverslips were filled with 500 µl 1X DPBS. On the other hand, the empty wells were filled with dH_2O in order to maintain humidity. Then, the plate was covered with parafilm and labeled properly. Until staining, the plate was stored at 4°C.

2.10.2 Staining

After the coverslips were washed with 1X DPBS one more time, they were rinsed for 60% isopropanol then incubated with freshly prepared oil red o (Sigma Aldrich, #MAK194) for 15 minutes in the dark. After that, the coverslips were rinsed with 60 % isopropanol, tap water, and DPBS, respectively. Oil red o-stained coverslips further permeabilized by using 1X DPBS with 0.1% Triton X-100 for 10 minutes. Then the coverslips were washed again and blocked for 20 minutes with SuperBlock (ScyTek, USA). While coverslips were being blocked, anti-StAR antibody solution was prepared in 1:100 dilution in the same superblock reagent. On a cell culture plate, parafilm was placed and labeled accordingly. Then, approximately 15 µl of antibody solution was placed onto the parafilm for every coverslip and they were placed onto the antibody droplets. After this process, some wet tissues were placed inside the plate without disrupting the coverslips for maintaining humidity. Then the plate was surrounded by parafilm and incubated overnight at 4^{o}C. On the other day, coverslips were washed by using 1X DPBS with 0.1% Tween-20 and then incubated with secondary antibodies prepared in the same blocking reagent in 1:100 dilution for nearly 1.5 hours at 37^{o}C. Afterward, the coverslips were washed carefully and placed onto approximately 7 µl of Fluoroshield mounting medium with DAPI (Abcam, UK) on a properly labeled microscope slide and after dried for a time, they were placed +4^{o}C until imaging. The images were taken by using an Olympus IX71 microscope.

2.11 Statistical Analysis

Hormone levels were expressed as mean +/- standard deviation. ANOVA and Bonferroni's multiple comparisons test were used to compare the estrogen and progesterone production of untreated, 10 IU hCG, 25 ng/ml Activin-A and 50 ng/ml rh-BMP7 treated HLGCs. For analysis, the GraphPad Prism 8.0.2 program was used. The data which has a p-value less than 0.01 was considered significant.

Chapter 3: **RESULTS**

3.1 Characterization of HLGCs

Since they undergo terminal differentiation after luteinization, human luteinized granulosa cells do not proliferate and they produce a high amount of progesterone and estradiol in the culture environment (Bildik et al., 2019). Therefore, their steroid content is higher than proliferating granulosa cells which was shown by staining with oil red o.

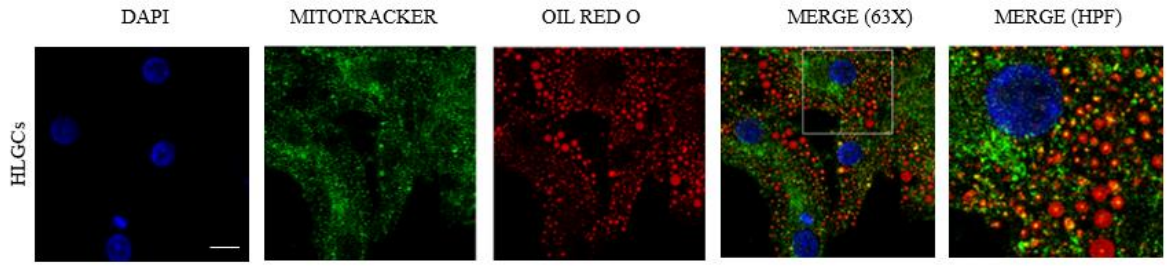

Figure 11: Confocal image of HLGCs stained with mitotracker and oil red o which shows mitochondria and steroid content of these cells as their characteristic feature (scale bar: 10 µm)

3.2 Expression of BMP7 Receptors on HLGCs

For annealing temperature optimization, the gradient PCR method was applied for ACVR1, BMPR1A, BMPR1B, and BMPR2 receptors.

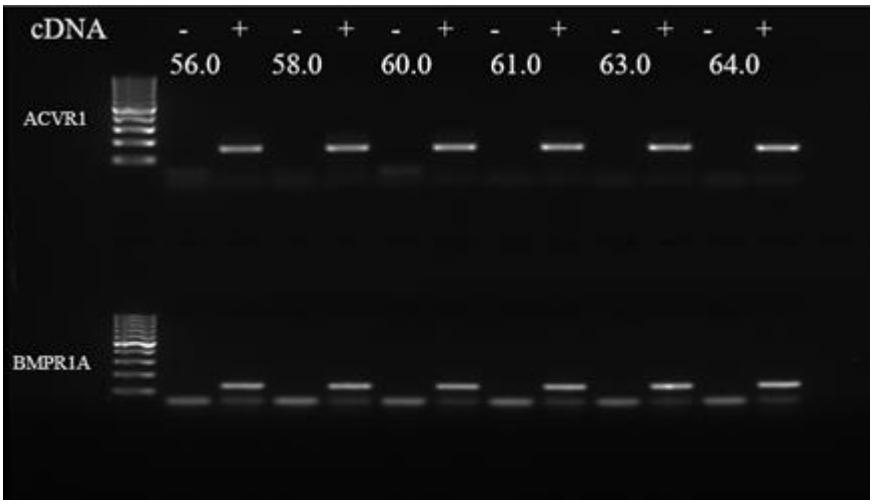

Figure 12: Gel image of gradient PCR of ACVR1 and BMPR1A

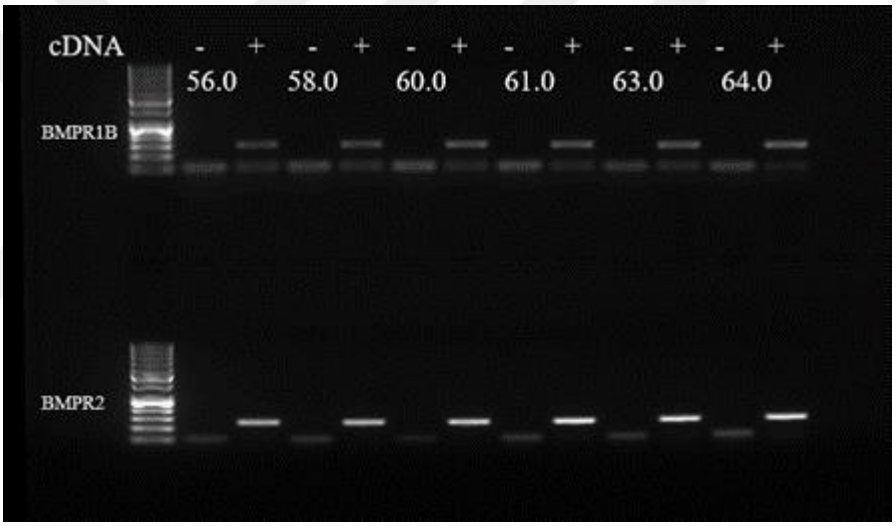

Figure 13: Gel image of gradient PCR of BMPR1B and BMPR2

The expression of BMP-7 receptors in the human luteal granulosa cells was validated with the qRT-PCR method. The data were normalized to GAPDH expression.

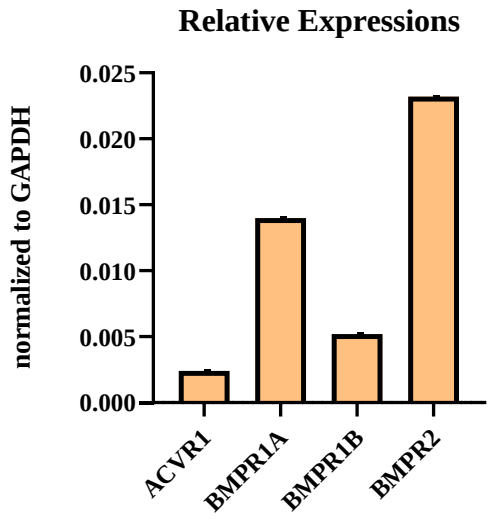

Figure 14: Relative expressions of BMP7 receptors on untreated HLGCs normalized to GAPDH

3.3 The Effect of BMP7 on Steroidogenic Function of HLGCs

Once the expression of BMP receptors on HLGC was confirmed with RT-PCR, the cells were treated with recombinant BMP-7 at the indicated concentrations in order to see its effects on steroidogenic function in comparison to luteotropic hCG and luteolytic activin-A treatments. In immunoblot analysis, BMP-7 and Activin-A treatments resulted in a significant decline in StAR expression whereas hCG did the opposite action. hCG slightly increased aromatase expression. However, such a notable change was not observed after treatment with BMP-7 or activin-A treatments (Figure 15).

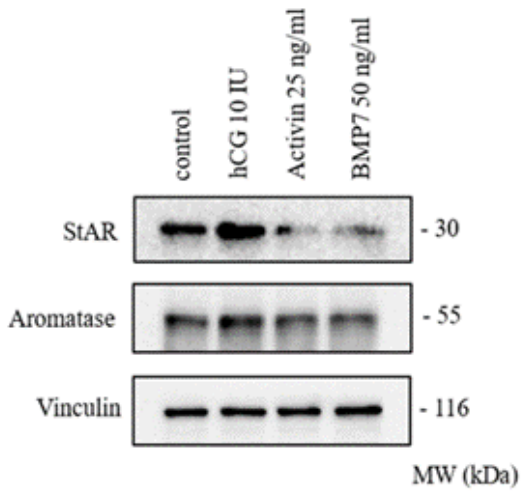

Figure 15: Immunoblot analysis of HLGCs. Cells were treated with 10 IU hCG, 25 ng/ml Activin-A and 50 ng/ml rh-BMP7, respectively. hCG treatment increased StAR expression whereas both Activin-A and rh-BMP7 treatments decreased it. While aromatase expression was slightly increasing with hCG treatment, it did not show any notable change with Activin-A and rh-BMP7 treatments.

3.4 E2 and P4 Production of HLGCs after treatment with hCG, Activin-A and rh-BMP7

In line with immunoblot results, recombinant human BMP-7 and Activin-A significantly reduced P_4 output whereas luteotropic hCG caused a robust increase in HLGCs. There were no significant differences in E_2 production among treatment groups (Figure 16).

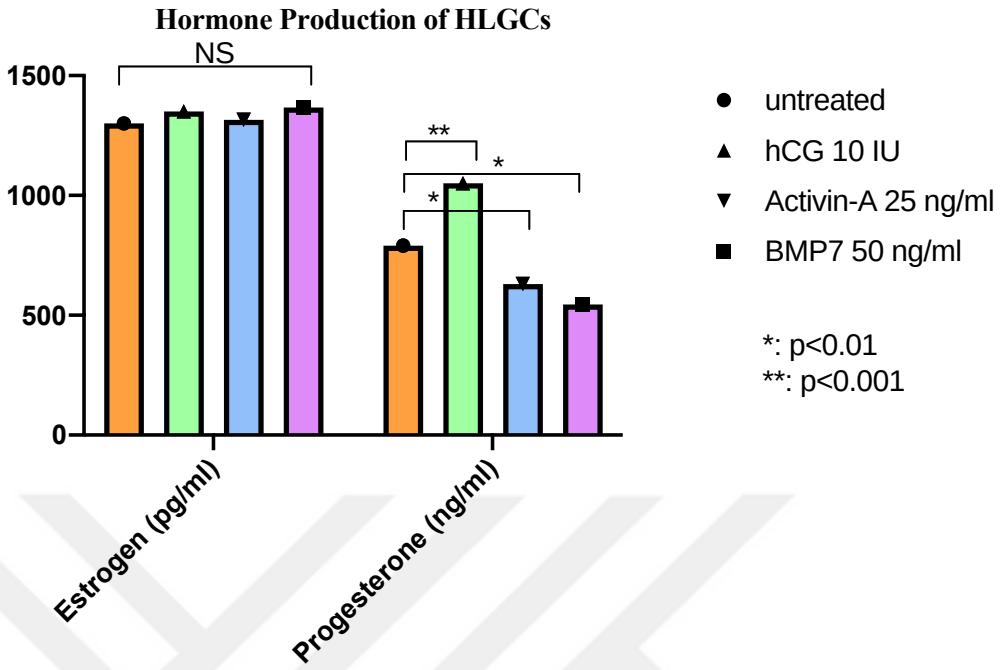

Figure 16: Hormone production of HLGCs after 10 IU hCG, 25 ng/ml Activin-A and 50 ng/ml rh-BMP7 treatments for 24 hours. While hCG treatment was significantly increasing the progesterone production, both Activin-A and rh-BMP7 treatment resulted in a significant decrease in it. However, there was no significant difference in estrogen production among the groups.

3.5 Immunofluorescence Staining of HLGCs with StAR antibody and oil red o

In parallel with the immunoblot analysis results, immunofluorescence staining of HLGCs also showed that both Activin-A and rh-BMP7 treatments decreased the number of the cells stained positively with StAR antibody with respect to the untreated and hCG-treated groups which indicate the decrease in StAR expression in these cells. In addition to StAR expression, oil red o staining showed that the size of lipid droplets also changed with hCG, Activin-A, and rh-BMP7 treatments. While the size of the droplets was increasing with hCG treatment, both Activin-A and BMP7 treatments caused a decrease in their sizes, respectively.

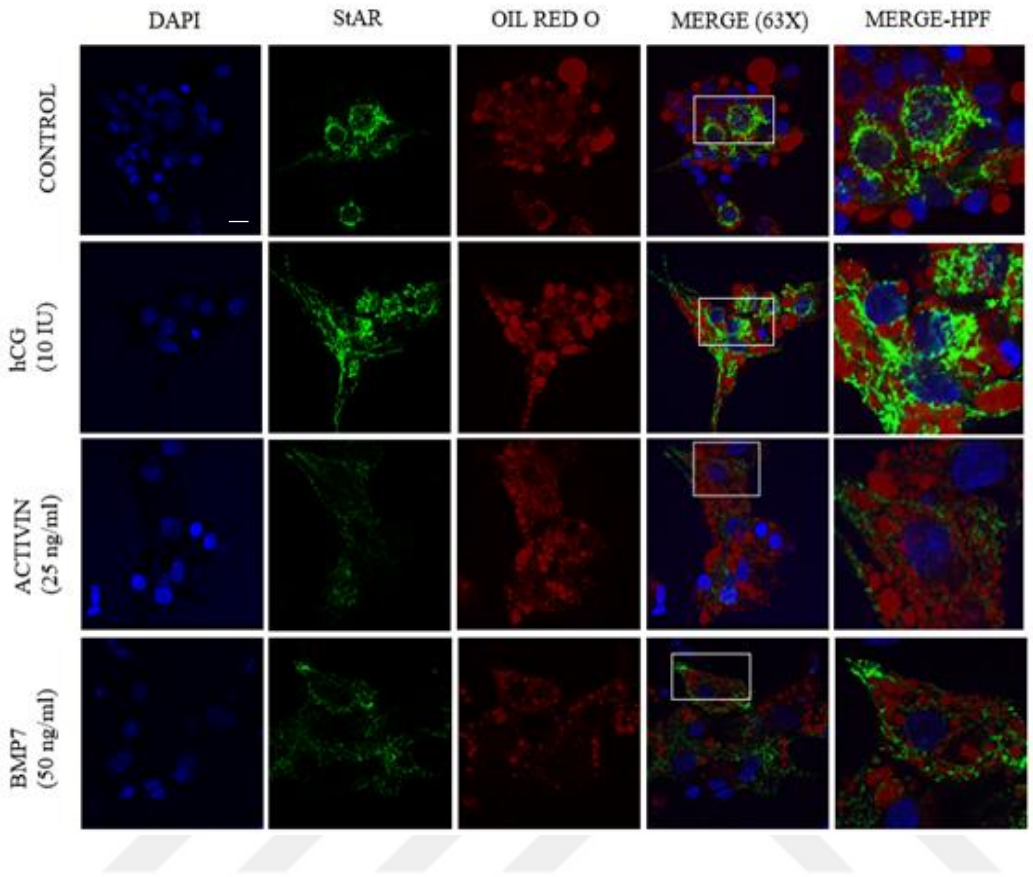

Figure 17: Confocal image of HLGCs stained with oil red o and StAR antibody. As hCG treatment increased the expression of StAR and size of the lipid droplets, both Activin-A and rh-BMP7 treatments decreased the expression of StAR and size of the lipid droplets in the HLGCs with respect to the untreated (control) group (scale bar: 10 µm)

Chapter 4: **DISCUSSION**

The process of luteinization which comprises morphological and biological remodeling of granulosa and theca cells after LH surge is one of the major events in the female reproductive system (Shrestha et al., 2019a). During luteinization, granulosa cells lose their proliferating ability and undergo terminal differentiation by downregulating cyclin D2 and upregulating cell cycle inhibitors p21Cip1 and p27Kip1 (Murphy, 2004). After luteinization, luteinized granulosa cells start to produce high amounts of progesterone to support endometrium for a possible pregnancy which causes an increase in the expression of the StAR enzyme which has a role in cholesterol mobilization and it is the rate-limiting enzyme during steroidogenesis (Miller, 2017b). There are some characteristic biological phenomena associated with luteinization such as tissue remodeling, functional and morphological changes in follicular cells, angiogenesis, and differential regulation of genes associated with proliferation (Russell & Robker, 2007). On the other hand, the demise of corpus luteum is called luteolysis which comprises functional loss of luteinized follicular cells, decreased production of progesterone, increase in cell death, breakdown of corpus luteum tissue, and macrophage infiltration (Devoto et al., 2009).

Understanding the luteal function is essential especially for the patients who undergo assisted reproduction. If implantation occurs during a natural cycle, the syncytiotrophoblasts of the implanting embryo will secrete β-HCG which stimulates the corpus luteum to continue synthesizing progesterone to support the endometrium and prevent miscarriages until the placenta takes over the duty of progesterone synthesis (van der Linden et al., 2015). On the other hand, the luteal function of the patients who are stimulated during assisted reproduction (IVF) is different from the natural cycle and it is known that corpus luteum functions are defective in these patients (Fatemi, 2009). The possible cause of this defect is multifollicular development in the ovary after stimulation. For these patients, luteal phase support (administration of medication) is essential to avoid miscarriages and increase the pregnancy rate. hCG and progesterone are the hormones used for luteal phase support during assisted reproduction. Studies show that hCG administration causes a significant increase in the rate of hyperstimulation with respect to the other luteal phase support agents (Fatemi et al., 2007).

One of the major risks of ovarian stimulation is ovarian hyperstimulation syndrome (OHSS) which is described by enlargement of ovaries, ovarian neoangiogenesis, the shift of fluid due to an increase in permeability of capillaries and some of the clinical features of OHSS are severe abdominal pain, vomiting, hypotension, electrolyte imbalance (Kumar et al., 2011; Bildik et al., 2019). Currently, instead of hCG, GnRH agonists are being used to avoid the possibility of OHSS during assisted reproduction. Therefore, the current studies about preventing OHSS are canalizing to find an agent which has the opposite effect of hCG. In a previously published study, it is shown that Activin-A which is a TGF-β family member reverses the luteinization profile of HLGCs and can be used for clinical trials in the future to prevent OHSS (Bildik et al., 2020). Therefore, in this study, both hCG which is known as luteal phase support and increase steroidogenesis in HLGCs (Bildik et al., 2020) and Activin-A is used to determine the effects of BMP7 on steroidogenesis in HLGCs.

As a beginning, BMP7 receptor expressions were confirmed on HLGCs by using the qRT-PCR technique. The optimum annealing temperature for each primer was decided as approximately 60^{o}C as can be seen from figures 12&13 by using the gradient PCR technique. In order to prevent dimerization of the primers, their concentrations might be diminished. Moreover, their relative expressions were normalized to GAPDH and displayed in figure 14 as it is stated in several articles that granulosa cells express BMP7 receptors (Silva et al., 2005; Rajesh et al., 2018). Since it is shown in previous articles that these cells are expressing LH and Activin-A receptors (Bildik et al., 2020; Bildik et al., 2020), after confirming that these cells are also expressing BMP7 receptors, they were treated with rh-BMP7. The optimum dose of BMP7 was chosen according to the literature, it was stated that 50 ng/ml rh-BMP7 is sufficient for granulosa cells to alter the gene expression and show its activity (Shimizu et al., 2012; Zhang et al., 2015).

In immunoblotting results, hCG increased the StAR expression which is the rate-limiting enzyme of steroidogenesis and progesterone production. Moreover, Activin-A treatment caused a decrease in StAR expression and progesterone production as expected. When the expression of StAR was compared to the untreated, hCG and Activin-A treated groups, it was seen that with respect to untreated and hCG treated groups, the expression decreased, while with respect to the Activin-A group there was no big difference in StAR expression. These results suggested that rh-BMP7 treatment affects HLGCs as Activin-A which means that BMP7 can be used to reverse the luteinization profile of these cells.

However, there was no notable change in aromatase expression as can be seen from figure 15. In a previous study (Chang et al., 2014), it was found that aromatase activity increases with Activin-A treatment; however, consistent with the findings of Bildik *et al.* in 2020, it was found that both Activin-A and rh-BMP7 don't affect aromatase expression significantly at the translational level. Aromatase has a role in estrogen synthesis in the ovary and by the cAMP/PKA pathway FSH activates aromatase expression in granulosa cells (Stocco, 2008). There was a slight increase in aromatase expression with hCG treatment, but no remarkable change was seen in Activin-A and BMP7 treated groups.

At the hormonal level, estrogen production didn't notably change among the groups, whereas progesterone production had changed. When the cells were treated with hCG, their progesterone production significantly increased. On the other hand, with Activin-A and rh-BMP7 treatments, progesterone production was significantly reduced. The decrease in between the progesterone production of Activin-A and rh-BMP7 treated cells was not statistically significant. However, with respect to the control group, the decrease in progesterone production of both Activin- A and rh-BMP7 treated groups was statistically significant. Therefore, it can be concluded that rh-BMP7 has a similar effect on progesterone production of HLGCs with Activin-A as it was hypothesized. In figure 17, the confocal image of hCG, Activin-A, and BMP7 treated groups were shown. HLGCs were first stained with oil red o which stains the lipid droplets in the cells by being dissolved in fat since it is a fat-soluble dye. Then, these cells were incubated with StAR antibody to indicate their StAR expression. As can be seen from the figure, both StAR expression and the size of the lipid droplets increased with hCG treatment and decreased with both Activin-A and BMP7 treatments. Since it is known that Activin-A reverses the luteinization profile, according to both immunoblotting and immunofluorescence results, BMP7 might also have the same effect on HLGCs. Therefore, it can be used for clinical trials to reverse luteinization and induce luteolysis for some clinical circumstances in assisted reproduction as stated in this section before.

There were some limitations during this study. One of them was BMP7 levels of cultured granulosa cells. BMP7 is known as a theca-cell-derived protein and although we couldn't measure its level on untreated cells, it still decreased the progesterone production. Another limitation was the variety of patients such as differences in their ages, triggers, follicle numbers, and collection method of follicular fluid. These factors affected the experiments, especially the quality of the cultured cells. The cells with lots

of erythrocytes were more prone to contaminations during culturing. In addition to these technical limitations, there was also a limitation about the stages of the corpus luteum. Since the corpus luteum has different stages during its formation and degradation, these results might not represent the late stages of a corpus luteum structure. Moreover, these results represented the effects of rh-BMP7 on steroidogenesis in HLGCs in vitro; therefore, it might not be true for a real in vivo environment.

Chapter 5: **CONCLUSION**

In conclusion, as Activin-A, rh-BMP7 reverses luteinization and downregulates the steroidogenic activity of human granulosa cells after luteinization. According to these results, rh-BMP7 might be tested in vivo, and its effects on steroidogenesis might be used for developing new strategies against OHSS. This data can be used for clinical trials in the future.

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